Auxin and Gibberellins in *Eucalyptus globulus*

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Abstract

Auxin and gibberellin (GA) are both crucial regulators of growth in the vascular cambium, effecting elongation, differentiation and the rate of cell division in trees. In several herbaceous species auxin has been found to influence the biosynthesis of GAs. To investigate the possibility of a similar interaction in *Eucalyptus globulus,* auxin levels were manipulated by treatment and application and GA levels monitored in the internodes of seedlings and the vascular cambium of trees. In seedlings, auxin reduction treatments typically reduced IAA by 10-fold or more and application of exogenous auxin restored auxin levels to levels similar to untreated plants. The effect of auxin reduction treatment on GAs was found to have its greatest impact on levels of GA_{20}, whilst GA_{19} was unaffected. Application of exogenous auxin increased GA levels in seedlings, but did not restore them. Auxin levels were reduced below the girdle in trees but application of auxin had no effect on GA levels in the vascular cambium. Further, analysis of the metabolites of radio-labelled GAs fed to excised seedling stems in MS media showed no effect of auxin on GA metabolism. These results suggest that while auxin can affect GA content in primary tissues, it does not necessarily affect GA content in tree stems.

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General Introduction

Eucalyptus globulus is a fast growing temperate hardwood species native to South Eastern Australia. It is grown as a wood fibre crop both in Australia and around the world. The plant growth regulatory substances indole-3-acetic acid (IAA) and gibberellins (GAs) have been shown to interact both in promoting growth and in the regulation and transport of themselves and each other in herbaceous species (Brian and Hemming 1958; Ross *et al.* 1995; Collett *et al.* 2000; Ross *et al.* 2000; O'Neill and Ross 2002; Ross *et al.* 2002; Swarup *et al.* 2002; Fu and Harberd 2003; Desgagne-Penix *et al.* 2005; Bjorklund *et al.* 2007). Both of these classes of phyto-hormone have been previously detected in tree species, including *E. globulus* (Hasan *et al.* 1994; Ridoutt *et al.* 1995), and both have been shown to influence the growth and development of wood (Wareing 1958; Wareing *et al.* 1964; Digby and Wareing 1966; Wang *et al.* 1992; Sundberg *et al.* 1994; Wang *et al.* 1995; 1997; Kalev and Aloni 1998; Eriksson *et al.* 2000; Druart *et al.* 2007).

The vascular cambium

The vascular cambium is a thin layer of meristematic tissue found between the xylem and phloem. Cambial cells can be found in two orientations, fusiform initials which form secondary xylem and phloem, and ray initials which form rays. Fusiform initials divide periclinaly; those on the interior side of the vascular cambium differentiate into xylem elements, those on the exterior side into phloem. Activity in the vascular cambium is seasonal in most species, with the growth rate and size of differentiated xylem elements changing throughout the year. Plant growth substances are thought to regulate many of the processes in this region including growth rates, dormancy control and differentiation.

Auxin

Auxin is both a promoter and regulator of plant growth and is known to interact with GAs, abscisic acid, brassinosteroids, jasmonic acid, ethylene and cytokinin (Swarup *et al.* 2002). Auxin has also been implicated in the regulation of plant growth in response to light and gravity, vascular patterning and differentiation, the control of dominance among shoot apices, the initiation of root meristem and organization of meristems (Berleth and Sachs 2001). A good general model of auxin's role is that it acts as a coordinating signal within the shoot, and from the shoot to the roots.

The predominant bioactive auxin in higher plants is indole-3-acetic acid (Hopkins 1999). Other endogenous auxins include indole-3-butyric acid (IBA), 4-Chloro indole-3-acetic acid (4-Cl-IAA), and phenylacetic acid (PAA). There are also several synthetic auxins; the most common of these are 1-naphthalacetic acid (NAA) and 2,4 dichlorophenoxyacetic acid (2,4-D), although there are also many other compounds similar to these two that also have auxin activity. Synthetic auxins are often used in agriculture as they are more chemically stable and are resistant to oxidation by the enzymes that normally break down IAA (Hopkins 1999).

In developing stems it is thought that auxin is the primary signal for undifferentiated stem tissue to form lateral organs such as leaf primordia. Application of IAA transport inhibitors has been shown to inhibit the formation of leaf primordia, and micro application of IAA to treated stems restores leaf formation in herbaceous species (Reinhardt *et al.* 2000). In trees, partial girdling substantially increased the number of epicormic buds that grew out below the site of the girdle, compared to sites above the girdle or un-girdled trees (Wignall *et al.* 1987). However, IAA is probably not the only factor controlling apical dominance (Morris *et al.* 2005), although these other factors are yet to be identified.

Auxin is transported both in the phloem and by an intercellular basipetal transport mediated system by auxin specific influx and efflux carriers in the cell membrane (Lachaud and Bonnemain 1984; Sundberg and Uggla 1998). The basipetal direction of inter-cellular auxin transport is maintained at least in part by the polar localisation of influx and efflux carriers at the respective ends of the cell (Jacobs and Gilbert 1983; Leyser1999; Friml and Palme 2002).

According to the chemiosmotic model of IAA transport (Rubery and Sheldrake 1974; Raven 1975; Goldsmith 1977) 15-20 % of the IAA present in the apoplast is in the protonated IAAH form and is able to enter cells either by diffusion or through carrier proteins. Once it enters the cell the higher pH de-protonates IAA and the resultant form is only able to leave the cell through a specialized carrier. IAA efflux carriers are localised in the cell membrane according to the direction of flow, resulting in polar transport of IAA. There is some uncertainty as to the relative importance of influx carriers in IAA transport but it has been suggested by Kramer and Bennett (2006) that IAA influx carriers are responsible for as much as 10-15 times more IAA uptake than diffusion alone in some cell types.

Both the stem apex and leaves are likely to be sources of auxin in tree stems. Decapitation has been shown to lower stem auxin content in *Pinus sylvestris* (Wang *et al.* 1997), *E. globulus* (McElwee 2003) and hybrid aspen (*Populus tremula* x *tremuloides* unless otherwise noted; Bjorklund *et al.* 2007) while defoliation has been shown to reduce stem auxin content in *P. sylvestris* (Wang *et al.* 1997) and *E. globulus* (McElwee 2003). Leaves have also been shown to be a source of IAA in pea (Jager *et al.* 2007) and auxin applied to mature leaves has been shown to move through the phloem and into the basipetal transport stream (Cambridge and Morris 1996).

It has also been suggested by Sundberg and Uggla (1998) that the vascular cambium may also have some capacity for de-novo synthesis of IAA as [$^{13}C_6$]IAA applied to the cut stump of decapitated pine seedlings was found to be progressively diluted with [^{12}C]IAA down the length of the stem in both intact and defoliated plants. However, the capacity to synthesize IAA was restricted to 1 year old stems, and in these [^{12}C]IAA only accounted for 10-20 % of total IAA present. It was concluded that the shoot apex was the major source of IAA found in the stem (Sundberg and Uggla 1998).

Radial gradients of IAA across the vascular cambium have been reported in both *P. sylvestris* (Uggla *et al.* 1996) and hybrid aspen (Tuominen *et al.* 1997). The gradient consists of a peak centred in the vascular cambium, tapering off to both sides, but more sharply into the phloem. It has been proposed that this radial gradient acts as a positional signal for developing xylem elements and that the width of the gradient, not the concentration of IAA, is the determining factor controlling growth (Uggla *et al.* 1998; Tuominen *et al.* 2000).

The concentration of IAA in the cambial region and developing xylem suggests that IAA transport in trees is through these tissues. This is supported by the findings of Lachaud and Bonnemain (1984) that the cambial region was the predominant tissue involved in the transport of exogenous [^{3}H]IAA in stems of *Fagus sylvatica* L. Further, in *Arabidopsis*, *AtPIN1* (an auxin efflux carrier gene) has been shown to be concentrated in vascular tissues (Galweiler *et al.* 1998). Girdling of shoots of *Abies balsamea* has also been shown to interrupt the transport of exogenous auxin (Little 1981).

In trees, Schrader *et al.* (2003) monitored the expression of several putative auxin transport genes across a radial transect of hybrid aspen trunk. Of the 6 genes studied, 2 were strongly expressed between the vascular cambium and the expanding xylem, while another 3 were expressed in that zone but had

similar or stronger expression between the expanding xylem and the zone of secondary wall thickening (more mature xylem). Analysis of the same genes on an apical/basal axis suggested that all of these genes had roles in the initiation and maintenance of vascular tissue at various stages of growth in different organs. Five of these six genes were auxin-inducible in auxin-deficient tissue, while the sixth was unregulated in the absence of auxin and not affected by auxin application (Schrader *et al.* 2003). Analysis of these six genes over the course of a year in "wild" trees showed that all were affected by season and were most strongly expressed during the growing season (May-July, Northern Hemisphere).

Tuominen *et al.* (1995) transformed hybrid aspen with the *iaaM* and *iaaH* IAA biosynthesis genes from *Agrobacterium tumefaciens* linked to the *mas 1'* promoter and reported reduced growth and a degree of disorganisation in the vascular cambium. There were only minor differences in the levels of both active and conjugated IAA in either the apical region or the mature stem compared to wild type plants.

A subsequent experiment by Tuominen *et al.* (2000) transformed hybrid aspen with the rate-limiting IAA biosynthesis gene (*iaaM*) from bacteria linked to the *rolC* cambium specific promoter, and reported an increase of 35-40 % in IAA content in the cambium. No significant changes in cambial growth or development were found. It was suggested that the radial distribution of auxin is more important as a regulator of cambial growth than the absolute concentration, although it must be noted that the change in IAA level was moderate compared to the changes in GA levels (2000 % increase compared with wild type; Eriksson *et al.* 2000) that have been studied in this species.

Taken together, it would seem as if altering IAA levels might provide an avenue for modulating aspects of wood quality, but it is unlikely to provide any means of increasing growth rates. Increasing GA concentrations in trees has been shown to have beneficial effects for both wood quality and growth rates.

Gibberellins

GAs have been associated with a variety of developmental processes and environmental responses in trees. Biosynthesis of GAs has been shown to be influenced by day-length (Moritz 1995; Eriksson and Moritz 2002) while lowering GA content has been shown to induce precocious and abundant flowering in both *E. globulus* and *E. nitens* (Griffin *et al.* 1992; Reid *et al.* 1995; Hasan and Reid 1995; Moncur and Hasan 1994). These effects were shown to be reversible by application of (the bioactive) GA_3 in *E. nitens* (Moncur and Hasan 1994). Decreased GA content has also been associated with a decrease in vegetative growth in trees (Griffin *et al.* 1992) as well as seedling height (Hetherington and Jones 1990). In addition to promoting longitudinal growth, application of GAs has also been suggested to promote phloem production (Wang *et al.* 1997).

The later stages of GA biosynthesis (Figure 1) consist of successive oxidations of C20 and its eventual removal. The resultant C19 GA is then 3-oxidised to one of the bioactive GAs, GA_1, GA_3 or GA_4. The oxidation and removal of C20 is carried out by the GA 20-oxidase enzyme family. Single GA 20-oxidase enzymes have been shown to catalyse all of these 20-oxidations *in vitro*. However, the differential response of GA 20-oxidase-encoding genes to decapitation and auxin treatments in hybrid aspen (Bjorklund *et al.* 2007) and *Arabidopsis* (Frigerio *et al.* 2006) suggest that individual GA 20-oxidase genes might be specific for certain reactions *in vivo*.

An "optional" 13-hydroxylation step can be applied to most active GA precursors, although GA_{12} and, in some species, GA_4 and GA_9, seem to be the preferred substrates for this reaction (Hedden and Kamiya 1997). 13-hydroxylation of GA precursors or the bioactive GA_4 produces GA_1, although direct deactivation of GA_{20} to GA_{29} is also possible. In *E. globulus,* it has been suggested that the early 13-hydroxylation pathway is dominant and that GA_1 is the predominant bioactive GA (Hasan *et al.* 1994; Ridoutt and Pharis 1998).

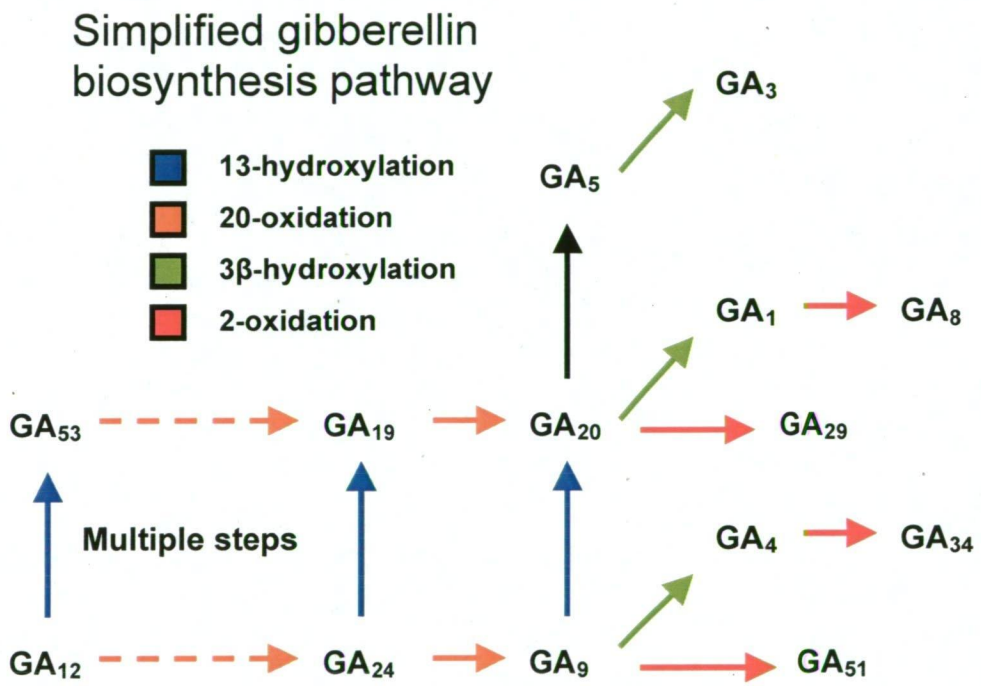


Figure 1.

Simplified gibberellin biosynthesis diagram showing the later stages of the formation of bioactive gibberellins and their de-activation.

Levels of active GAs can be regulated by both feed-forward and feed-back regulation of the steps in these pathways (Hedden and Kamiya 1997; Ross *et al.* 1999; Elliott *et al.* 2001) as well as by external factors such as other growth hormones and environmental conditions (Hedden and Kamiya 1997; Yamaguchi and Kamiya 2000). The expression of *PttGA20ox1* has been shown to be affected by day length in hybrid aspen (Eriksson and Moritz 2002) and it has been inferred that 3-oxidation is also reduced by short days (Moritz 1995). Eriksson and Moritz (2002) also reported a negative feedback response of the expression of *GA20ox1* when GA_4 was applied to hybrid aspen.

Eriksson and Moritz (2002) also monitored the spatial expression of *GA20ox1* in hybrid aspen seedlings, both along the axis of the plants in the stem, leaves and petioles, and also the radial distribution from the phloem to the mature xylem. Levels of expression were generally low except in late expanding internodes and leaf blades. This is in contrast to the sites found to have the highest levels of bioactive GAs, namely the early expanding tissues and the apical region. It was hypothesised that this discrepancy may be due to the transport of GA_{20}, feedback regulatory effects, or a simple lack of a strong relationship between transcript level and enzymatic activity due to either post transcriptional modifications or substrate availability (Eriksson and Moritz 2002).

GAs have previously been associated with increased rates of cambial cell division, cell elongation and promotion of phloem formation at high GA/IAA ratios (Wareing 1958; Wareing *et al.* 1964; Digby and Wareing 1966; Wang *et al.* 1997). Isrealsson *et al.* (2005) identified the presence of radial gradients across the vascular cambium for bioactive GAs and their immediate precursors and metabolites. The peak concentration of bioactive GA_1 and GA_4 was reported to be in the developing xylem and the peak for the immediate precursors of these GAs (GA_{20} and GA_9) to be in the phloem.

A small secondary peak of GA_{20} and GA_9 was also present in the region of expanding xylem. The metabolites of the bioactive GAs were found in highest abundance in the developing xylem but were also present in the phloem.

Isrealsson *et al.* (2005) also monitored the expression of GA biosynthesis and deactivation genes across the cambium, using RT-PCR. The expression profile of *PttGA20ox1*, which synthesises the rate-limiting step in the later stages of bioactive GA biosynthesis (Eriksson *et al.* 2000; Isrealsson *et al.* 2004), was found to be predominantly expressed in the developing xylem. This supports the hypothesis that bioactive GAs are likely to be synthesised *in situ*. However, expression of *PttGA20ox4* and *PttGA3ox1*, the products of which catalyse the penultimate and final steps in the GA biosynthetic pathway respectively, were found to be highest in the phloem. It was suggested that the higher concentration of *PttGA20ox4* in the phloem may be due to feedback loops regulating this gene in response to the high and low levels of bioactive GA found in these tissues respectively (Isrealsson *et al.* 2005). However, in decapitated seedling stems, Bjorklund *et al.* (2007) found no down-regulation of *PttGA20ox4* when exogenous GA was applied. It is also possible that *PttGA20ox4* is preferentially expressed in this region to perform earlier steps in the GA biosynthesis pathway, which are also known to be catalysed by GA 20-oxidase genes, such as the conversion of GA_{19} to GA_{20}. However, any specificity or substrate preference for either of these enzymes is yet to be determined.

In addition to the expression of GA biosynthesis genes, Isrealsson *et al.* (2005) also examined the expression of the GA signalling and response genes, *DELLA-like1* and *GIP-like1* respectively, in the zone of expanding xylem. Both of these genes had a clear peak in expression in the zone of expanding xylem, further supporting the hypothesis that GAs are active in this zone.

Application of GA biosynthesis inhibitors has been shown to impact on the growth of several tree species. Paclobutrazol, an inhibitor of early GA biosynthesis (Dalziel and Lawrence 1984), was found to reduce both seedling height and internode length when applied to seedlings of *E. globulus* (Hetherington and Jones 1990). Injection of the GA biosynthesis inhibitor Trinexpac-ethyl (an acylcyclohexanedione type inhibitor of GA3β-hydroxylase action, Rademacher *et al.* 1992) into the stems of 3 year old (6.5 m tall) *E. globulus* trees reduces levels of both GA_{20} and GA_1 as well as fibre length after 7 weeks (Ridoutt *et al.* 1996). Application of prohexadione (another acylcyclohexanedione type GA inhibitor) to 3 year old *P. sylvestris* seedling shoots was found to lower the levels of GA_1, GA_3 and GA_4 in the region the inhibitor was applied. Cambial growth and stem elongation were also inhibited in this region (Wang *et al.* 1995).

In addition to experiments where GAs are applied exogenously, both hybrid aspen and tobacco plants with altered expression of GA biosynthesis genes have been generated and described. Tobacco plants transformed with *GA 20-oxidase* from citrus (*CcGA20ox1*), under the control of the cauliflower 35S promoter, were found to be up to twice as tall as wild type plants and were unresponsive to exogenous application of GA_3. Stem lengths were reduced by application of paclobutrazol, and the effects of paclobutrazol were countered by application of GA_3 (Vidal *et al.* 2001). Further work with transgenic tobacco by Biemelt *et al.* (2004) produced plants expressing either *AtGA20ox* or *AtGA2ox* genes with the cauliflower 35S promoter. *AtGA20ox* over-expressing plants were taller and had higher stem weight than wild type, as well as a wider, more heavily lignified zone of secondary xylem. *AtGA2ox* over-expression had the opposite effect to *GA 20-oxidase* over-expression in regard to all of these traits.

In trees, Eriksson *et al.* (2000) produced transgenic lines of hybrid aspen expressing *AtGA20ox1* under the control of the cauliflower 35S promoter. Transgenic trees were found to have up to 24-fold higher levels of bioactive GA in the stem than wild type plants. Phenotypic changes included 64 % higher shoot weight, 126 % more dry stem weight and 71 % more xylem fibre cells that were approximately 8 % longer than wild type.

Busov *et al.* (2003) developed *a GA 2-oxidase* over-expressing mutant hybrid aspen (*Populus tremula* x *alba*) with a dwarf phenotype. Mutant plants were approximately 4-fold shorter than wild type plants while maintaining a similar number of internodes. Levels of GA_1 and GA_4 were substantially reduced in mutant plants (approximately 5-fold) while levels of deactivated GA (GA_{29}, GA_{34} and GA_8) were all elevated. Dwarfism was reversed by application of GA_3 (which is bioactive, but not metabolised by *GA 2-oxidase*). *Populus tremula* over-expressing *PttGA3-oxidase* were generated by Isrealsson *et al.* (2004) but showed no major changes in morphology. It was concluded that GA 3-oxidation is not rate-limiting in aspen.

Kalev and Aloni (1998) applied both IAA and GA to parenchyma in *Pinus pinea* hypocotyls. When GA was applied no tracheids were formed and when IAA was applied short tracheids re-differentiated. When both IAA and GA were applied tracheids formed that were approximately three times longer than those formed when only IAA was applied. Little and MacDonald (2003) reported very similar results when GAs were applied to the shoots of both *P. sylvestris* and *Picea glauca*. Stem elongation caused by application of GA was attributed to cellular elongation as the number of stem units was unaffected by treatment.

Together with the well documented relationship between GAs and the elongation of primary growth in both herbaceous species and in tree seedling stems, there is a solid relationship between GA content and cell elongation. It has been suggested several times that GAs may be actively transported in trees. In *P. sylvestris*, labelled GAs applied to the shoots of seedlings were later recovered from all parts of the plant and were readily metabolised (Wang *et al.* 1996). GAs have also been specifically isolated from the phloem in aspen and it was suggested that they may be transported both in the phloem, and from the phloem across the vascular cambium to the developing xylem (Isrealsson *et al.* 2005). It was also suggested by Bjorklund *et al.* (2007) that GA_4 applied to decapitated hybrid aspen seedlings is transported in a basipetal fashion.

Auxin/gibberellin interactions

Auxin has been shown to increase GA levels or promote GA biosynthesis genes in several herbaceous species including pea, rice, barley, tobacco and *Arabidopsis*. In rice, the male sterile Zhenshan 97A variety was identified as being IAA deficient in panicles and upper internodes. Down-regulation of *OsGA3ox2* and a corresponding drop in GA_1 levels was also reported in these plants (Yin *et al.* 2007). Application of triiodobenzoic acid (TIBA, an IAA transport inhibitor) or decapitation were found to substantially reduce IAA, *OsGA3ox2* and bioactive GA content in the stem, while expression of *OsGA2ox1* was increased when these treatments were applied. When 50 μM of IAA was applied to the cut stump *OsGA3ox2* expression and bioactive GA levels were increased and *OsGA2ox1* expression was decreased.

In tobacco shoots, decapitation has also been shown to reduce levels of IAA and bioactive GAs in the stem while application of IAA to the cut stump was found to restore GA levels (Wolbang and Ross 2001). In the same experimental system decapitation reduced conversion of [^{14}C] labelled GA precursors to bioactive GA and promoted deactivation of [^{14}C]GA_{20} to [^{14}C]GA_{29}.

In barley (*Hordeum vulgare*), removal of the inflorescence was found to reduce stem IAA levels as well as levels of bioactive GA_1 and GA_3 in both internodes and nodes below the site. Application of IAA to the stump recovered levels of bioactive GA in most cases. Conversion of [^{14}C]GA_{20} and [^{14}C]GA_5 to [^{14}C]GA_1 and [^{14}C]GA_3 respectively was also found to be reduced in decapitated plants, as was *GA 3-oxidase* transcript levels. These effects were recovered by auxin application to the cut stump (Wolbang *et al.* 2004).

Fu and Harberd (2003) reported that in *Arabidopsis* roots IAA modulated growth by altering cellular response to GAs. However, in pea roots it was concluded that auxin has a stronger effect on GA biosynthesis than on GA signalling (Weston *et al.* unpublished data 2007). Application of PCIB or yokonolide B (inhibitors of IAA action) to pea roots was found to down-regulate the expression of *PsGA20ox1* and to up-regulate both *PsGA2ox1* and *PsGA2ox2*. However, the effects of auxin action inhibitors are not always identical. In the presence of PCIB, expression of *PsGA3ox1* was up-regulated, while *PsGA3ox2* was down-regulated, but in the presence of yokonolide B both genes were down-regulated. In the presence of either of these inhibitors, overall 3-oxidation of labelled GA_{20} was reduced (Weston *et al.* unpublished data 2007).

The chemical inhibitors of IAA transport, TIBA and 1-*N*-naphthylpthalamic acid (NPA), as well paclobutrazol were applied to *Arabidopsis* plants (Desgagne-Penix *et al.* 2005). Expression of *AtGA20ox1* was increased by application of either IAA transport inhibitor or paclobutrazol. However, *AtGA20ox2* was only found to be substantially over-expressed when paclobutrazol was applied. In a separate experiment Frigerio *et al.* (2006) monitored the expression of different GA 20-oxidation, GA 3-oxidation and GA 2-oxidation genes in response to application of 50 μM of NAA. While some GA 20-oxidation and GA 2-oxidation genes were up-regulated and one *GA2ox* down-regulated by application of NAA, expression of other GA oxidation genes were unaffected.

Application of TIBA to pea stems was found to reduce turnover of applied [^{13}C,^{3}H]GA_{20} to [^{13}C,^{3}H]GA_1 below the site of application by Ross (1998). Further investigation of this effect revealed that decapitation reduced both IAA and GA_1 content in pea internodes and that application of IAA to the cut stump recovered GA_1 levels (Ross *et al.* 2000). Transcript levels for the gene *PsGA3ox1* were found to mirror those of GA_1 levels. In addition, levels of *PsGA2ox1* were increased by decapitation and reduced by IAA application (Ross *et al.* 2000; 2002; O'Neill and Ross 2002). In contrast to the down-regulation of *PsGA2ox1* by IAA, *PsGA2ox2* was found to be up-regulated when IAA was applied to decapitated plants (O'Neill and Ross 2002). It was hypothesised that up-regulation of *PsGA2ox2* may be due to feed-forward regulation of this gene by increased GA_1 content in IAA treated stems, as documented by Elliott *et al.* (2001). Additionally, growth associated with either auxin or GA in peas has been suggested to be dependant on the presence of the other (Brian and Hemming 1958; Ockerse 1970).

It has been suggested by Ross *et al.* (2002) that in respect to stem elongation, GA is actually a component of the IAA response pathway, and that at least some of the elongation observed in pea stems when IAA is applied is the result of increased GA_1 levels in these stems.

The effects of both IAA and GAs on the growth of cambial derivatives have also been studied in a number of tree-based experimental systems. Digby and Wareing (1966) demonstrated that an increased proportion of GA to IAA applied exogenously to undifferentiated vascular cambial tissue increased the proportion of phloem produced, whilst an increased proportion of IAA increased the production of xylem elements. Both hormones applied together had a synergistic effect on both cambial division and xylem production. Wang *et al.* (1997) linked decreased IAA content in the stem with a reduction in longitudinal growth as well as a reduction in both xylem and phloem production. When the same experimental system was made deficient in both IAA and GA, the application of GAs alone promoted longitudinal growth and phloem production whilst application of IAA and GA together was found to increase longitudinal growth as well as both xylem and phloem production.

Field (1974) reported that GAs may promote the transport of IAA in the xylem of *Salix fragilis* L. in lateral, tangential and longitudinal orientations. It was suggested that GAs may be promoting IAA transport by affecting cell membrane permeability. Bjorklund *et al.* (2007) also reported an increase in polar transport of [^{13}C]IAA applied to the cut stump of decapitated hybrid aspen when GA_4 was also applied. In this system, GA was shown to up-regulate the expression of *PttPIN1* (Bjorklund *et al.* 2007), which is the predominant polar auxin transport gene of the vascular cambium and expanding xylem of hybrid aspen (Schrader *et al.* 2003). *PttPIN1* is known to be up-regulated by IAA (Schrader *et al.* 2003; Vieten *et al.* 2005), but was also found to be up-regulated 50 % by GA application to decapitated seedlings when compared to the lanolin control. When IAA and GA were

applied together, *PttPIN1* expression was higher than in plants where only IAA was applied. Using the micro-array of Andersson *et al.* (2004), Bjorklund *et al.* (2007) also showed that 83 % of genes affected by GA treatment were also affected by IAA treatment in hybrid aspen, including 98 of the 100 genes most strongly expressed in response to GA treatment.

Application of auxin to decapitated hybrid aspen was found to promote the expression of *PttGA20ox1* and *PttGA20ox4* and to down-regulate the expression of *PttGA2ox1*. Expression of *PttGA2ox2* was found to be increased in decapitated plants and was reduced to a level of expression similar to intact plants by application of IAA (Bjorklund *et al.* 2007). The effects of IAA on GA levels were not as substantial. Application of IAA to decapitated seedlings did not have any effect on GA_1 levels after one or two days, or on GA_4 levels after one day. However, GA_4 levels in decapitated plants were found to be increased approximately 3-fold two days after IAA application. Contrary to herbaceous species, decapitation did not reduce levels of either GA_1 or GA_4 compared to intact plants in this experimental system (Bjorklund *et al.* 2007).

In this study we have focused on GA_1 and its immediate precursors/metabolites in the early 13-hydroxylation pathway.

$$GA_{19} \rightarrow GA_{20} \rightarrow GA_1 \rightarrow GA_8$$
$$\downarrow$$
$$GA_{29}$$

Hasan *et al.* (1994) identified and quantified GAs and IAA from the cambial region of *E. globulus* and suggested that the early 13-hydroxylation pathway was dominant in this species. Similar work by Ridoutt *et al.* (1995) agreed with this and added the suggestion that the early 13-non-hydroxylation pathway may also be operating, but further work by Ridoutt and Pharis

(1998) studying the metabolism of radio-labelled GA substrates *in situ* concluded that the early 13-hydroxylation pathway was likely to be dominant.

GAs have been shown to affect growth in *E. globulus*. Ridoutt *et al.* (1996) injected acylcyclohexanedione (a GA biosynthesis inhibitor) into the stem of *E. globulus* and reported both a reduction in levels of active GAs and fibre length. Williams *et al.* (1999) reported a similar finding when they treated *E. nitens* seedlings with a range of GA biosynthesis inhibitors, with seedling height, GA_{20} and GA_1 levels reduced.

E. globulus is difficult to transform (Serrano *et al.* 1996; Moralejo *et al.* 1998) and, as such in this species, there has not yet been any work undertaken analogous to that done on hybrid aspen. However, comparisons that can be made with the available data show remarkable similarity between hybrid aspen and *P. sylvestris*. That the hormone gradients discussed above are consistent between an angiosperm and a gymnosperm species indicates that these gradients are likely to be a highly conserved method of developing organised secondary tissue in trees. Therefore, it is thought likely that hormonal control of growth amongst tree species is not largely influenced by species and that similar hormone concentration gradients would exist in *E. globulus*.

Hypothesis

Auxins and GAs have been shown to have both individual and synergistic effects on cambial growth and differentiation as well as the subsequent development of cambial derivatives. GAs have been linked with cell elongation and the promotion of phloem formation while IAA has been associated with the promotion of xylem formation and its subsequent differentiation and development. These individual effects of auxin and GA are also generally enhanced in the presence of the other (Wareing 1958; Wareing *et al.* 1964; Digby and Wareing 1966; Wang *et al.* 1995; 1997; Kalev and Aloni 1998; Eriksson *et al.* 2000; Little and MacDonald 2003).

In several herbaceous species IAA regulates the levels of bioactive GAs (Ross *et al.* 2000; Elliot *et al.* 2001; Wolbang and Ross 2001; O'Neill and Ross 2002; Fu and Harberd 2003; Wolbang *et al.* 2004; Desgagne-Penix *et al.* 2006; Frigerio 2006; Yin *et al.* 2007) and it has also been shown to affect the expression of GA biosynthesis genes and GA levels in hybrid aspen (Bjorklund *et al.* 2007).

The aim of this project was to develop methods to alter auxin levels in the stems of *E. globulus* seedlings and trees and test the hypothesis that auxin levels influence the GA biosynthetic pathway in the stems of *E. globulus*.

General Methods

Both whole seedling stems and the vascular cambium of trees were examined in the following experiments. Trees were girdled at breast height (1.3 m above ground) while seedlings were decapitated and defoliated to reduce auxin content. For methodology specific to these experiments refer to the relevant chapter. Extraction and quantification of IAA from both systems was successful and relatively simple. However, the quantification of most GAs, especially the bioactive GA_1, from both seedlings stems and the vascular cambium of trees presented numerous technical challenges

Hormone Extraction

Both cambium and seedling stem segments were extracted in 80 % (v/v) MeOH containing 250 mg/L BHT at -20°C overnight then at 4°C for 24 hours. Seedling stems were macerated with a food processor and both types of sample were vacuum filtered using Whatman no.1 filter paper. Samples to be analysed for GAs had deuterated internal standards (kindly provided by Professor LN Mander, ANU, Canberra, Australia) added at approximately 1:1 ratio internal standard: endogenous on the basis of previous reported levels for the relevant tissue type (Hasan *et al.* 1994; Ridoutt at al 1995 for cambium, Williams *et al.* 1999; McElwee 2003 for seedlings).

In later experiments GA standards were added in amounts approximately equal to the endogenous GA levels found in this project. Internal standards for auxins were [$^{13}C_6$]IAA (Cambridge Isotope Laboratories, Andover, MA, USA) and where appropriate [$^{13}C_6$]2,4-D (Cat. No. XA11940 200AC, Dr. Ehrenstorfer Laboratories, Augsberg, Germany). All internal standards were dissolved in MeOH except for [$^{13}C_6$]2,4-D which was dissolved in acetone. Typical amounts of internal standard per sample added were as shown in Table 1.

Table 1

Typical amounts of deuterated or [^{13}C] internal standard added to extracts from different tissues examined in this project.

Seedlings	GA_{19}	GA_{20}	GA_1	GA_8	GA_{29}	IAA	
ng IS	10	1	1	10	1	50-600	
Cambium	GA_{19}	GA_{20}	GA_1	GA_8	GA_{29}	IAA	2,4-D
ng IS	10	2	2	10	2	10-100	5-200

Ranges are given where the amount of internal standard added varied depending on the treatment applied. Typical sample fresh weights were from 1-5 g for both seedling internodes and cambial scrapes. Samples were stored in sealed glass containers at -20°C until further processed.

20 000 dpm of [^{3}H]GA_{20} (approximately 7.5 Ci/mmol^{-1} at time of use, Amersham International, Buckinghamshire, UK) was added to each aliquot from the stored sample before it was reduced to near dryness by rotary evaporation in a water bath at 30°C. Samples were then resuspended in distilled water containing 0.4 % (v/v) acetic acid (AA) and loaded in three 1-2 ml washes onto 0.5 g (IAA and 2,4-D aliquots) or 2 g (GA aliquots) C18 Sep-Pac cartridges (Waters Corp. Milford, Mas. USA). Sep-Paks were preconditioned with 12 ml MeOH then 12 ml 0.4 % (v/v) AA, then washed with a further 1 ml 0.4 % AA and eluted with 12 ml 70 % MeOH/0.4 % AA dH_2O (v/v). Eluted extract was then taken to dryness by rotary evaporation and resuspended in 1 ml 20 % MeOH/0.4 % AA in dH_2O.

Free Acid HPLC

All GA and IAA samples were fractionated on High Performance Liquid Chromatography (HPLC, model 1525 binary HPLC pump and injector, 2487 dual λ absorbance detector, 10 cm x 8 mm i.d. 10 μm Radial-Pak C18 cartridge in a RCM 8 x 10 module, Waters Assoc. Milford, MA. USA) with an initial solvent mix of 20 % MeOH/0.4 % AA (v/v) on a linear gradient to 75 % MeOH/0.4 % AA (v/v) over 25 minutes with a flow rate of 2 ml/minute. Loading of the 2 ml injector coil was done with 3 washes of 800 μL of 20 % MeOH/0.4 % AA, with the extra 400 μL accounted for by the volume of a radial filter attached to the syringe. Fractions were collected at 1 minute intervals and 100 μL aliquots were taken and analysed for radioactivity by liquid scintillation counting as described above. Retention times of GAs and IAA were deduced from the retention time of $[^3H]GA_{20}$. Hormone-containing fractions were grouped and taken to dryness under vacuum, with the exception of GA_1, which was dried by rotary evaporation as described above due to concerns about its degradation when dry.

Processing of more than 2 g of tissue (as measured by fresh weight) was found to have serious adverse effects on chromatography in this system, broadening the peak of labelled GA tracer from 1-3 fractions to approximately 10. This was a similar effect to that reported by Hasan *et al.* (1994) who worked on much larger amounts of tissue per sample (as measured by fresh weight).

Methylation and Ether Partitioning

All samples were redissolved in 400 µL MeOH and methylated by addition of 1.5 ml diazomethane or, in later experiments, (trimethylsilyl) diazomethane (Sigma-Aldrich Co.). Samples were then sealed and allowed to stand for a minimum of 5 minutes before being placed in a heated block at 30°C and taken to dryness under a stream of dry nitrogen.

Gibberellins A_1, A_8 and A_{29} were redissolved in 1 ml dH_2O and partitioned 3 times against 600 µL di-ethyl ether. The ether fractions were then dried in a heat block at 30°C under a stream of nitrogen. Any residual water was removed by an additional 20 minutes of drying under a vacuum.

Methyl-Ester HPLC

Gibberellins A_1, A_8, and A_{29} were subject to an additional HPLC step (U6K injector, 10 cm x 8 mm i.d. 10 µm Radial-Pak C18 cartridge fitted in a RCM 8 x 10 module and two model 510 pumps, Waters Assoc. Milford, MA, USA) of 30 % MeOH to 60 % MeOH exponential gradient over 35 minutes. This was followed by 25 minutes of isocratic elution at 60 % MeOH; a flow rate of 1.6 ml/minute was used for both phases. Fractions were collected every minute and either [^{14}C]GA_3 (for GA_8 and GA_{29}, supplied by L.M. Mander, ANU, Canberra, Australia) or [^{3}H]GA_1 (for GA_1, specific activity approx. 11 Ci/mmol) were used as tracers.

The stock of [^{14}C]GA_3 was found to contain trace amounts of either unlabeled GA_1 or an impurity of identical retention time on GC-MS and was not suitable as a tracer for this GA. [^{3}H]GA_{29} was also trialled as a tracer for methyl-ester HPLC for GA_1 and was also found to contain putative unlabeled GA_1 as an impurity. [^{3}H]GA_1 was generated by feeding [^{3}H]GA_{20} to *E. coli* expressing *PsGA3ox1* with purification of labelled GA_1 by free acid HPLC as

described above. The specific activity of this substrate was found to be high enough such that the quantities necessary for use as a tracer in samples were undetectable on GC-MS.

100 µL aliquots were taken from each fraction and radio-counted as described above. Broad zones for each GA were grouped and taken to dryness under vacuum overnight: except for GA_1, which was dried by rotary evaporation as it was found to be susceptible to degradation when dry.

TCMS

All GA and IAA samples were dissolved in 10 µL pyridine and derivatised with 40 µL bis(trimethylsilyl)trifluriacetamide (BSTFA, Alltech, Deerfield, IL USA) at 80°C for 30 minutes, dried at 30°C under a stream of dry nitrogen, then re-derivatised with 20 µL BSTFA at 80°C for 15 minutes.

GC-MS

Two different gas chromatography-mass spectrometry (GC-MS) systems were used in the analysis of samples. The best results for GA_{20}, 2,4-D and IAA were obtained from GC-MS-MS as the fragmentation of these compounds and their ions suit them to the dual MS procedure (Figures 4, 5 and 6). GA_1 and GA_8 were not suited to this process as they do not fragment to any substantial degree in the MS. The un-fragmented molecular mass was used to quantify these GAs on a high resolution GC-MS system (Figures 2 and 8). GA_{19} was successfully run on either system with a single MS procedure (Figures 3 and 7).

High Resolution GC-MS

High resolution GC-MS was performed on a Hewlett-Packard 5890 Series II Gas Chromatograph using either a HP1 (25 m x 0.32 mm internal diameter, 0.17 μm film. Agilent, Santa Clara, CA) or a BPX-608 column (25 m x 0.32 mm internal diameter, 0.4 μm film. SGE, Ringwood, Victoria, Australia). Injection was in splitless mode and helium was used as a carrier gas at the rate of 2 ml/minute and 15 p.s.i. This was linked to a Kratos Concept ISQ Mass Spectrometer controlled by a Mach 3 data system. Perflurokerosene (PFK) was used as a reference compound to provide lock masses.

Gas chromatograph temperature profiles were 60-230°C @ 30°C/min then 3°C/min for GAs and IAA when run on the HP1 column. 60-250°C @ 30°C/min then 10, 6 or 3°C/min to 300°C were all used on the BPX-608 column for GA_1.

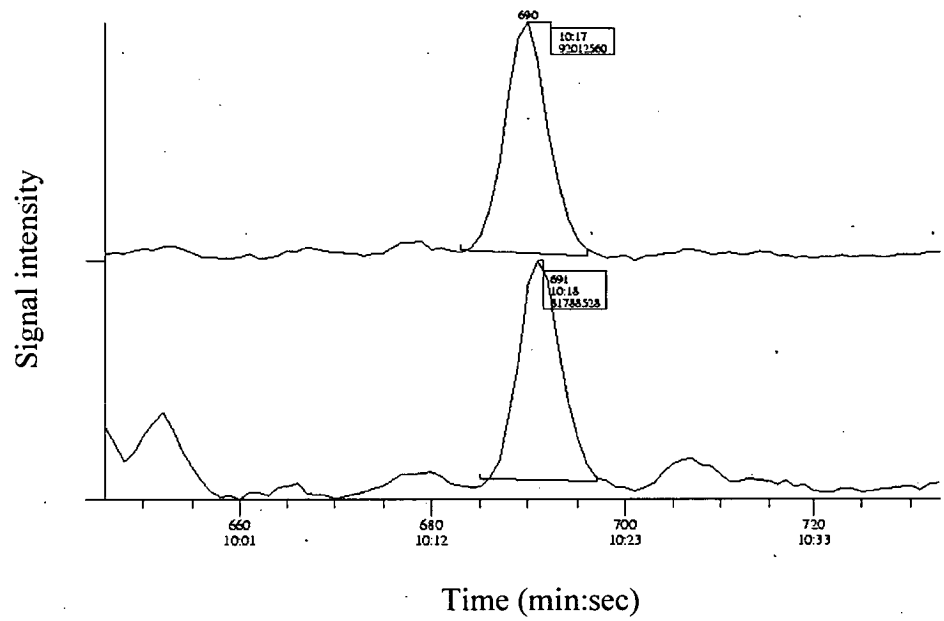


Figure 2.

Typical mass chromatograms of derivatised GA_8. Endogenous (lower) and $[^2H_2]GA_8$ (upper) have a slight offset due to the earlier retention time of the deuterated form on the GC column.

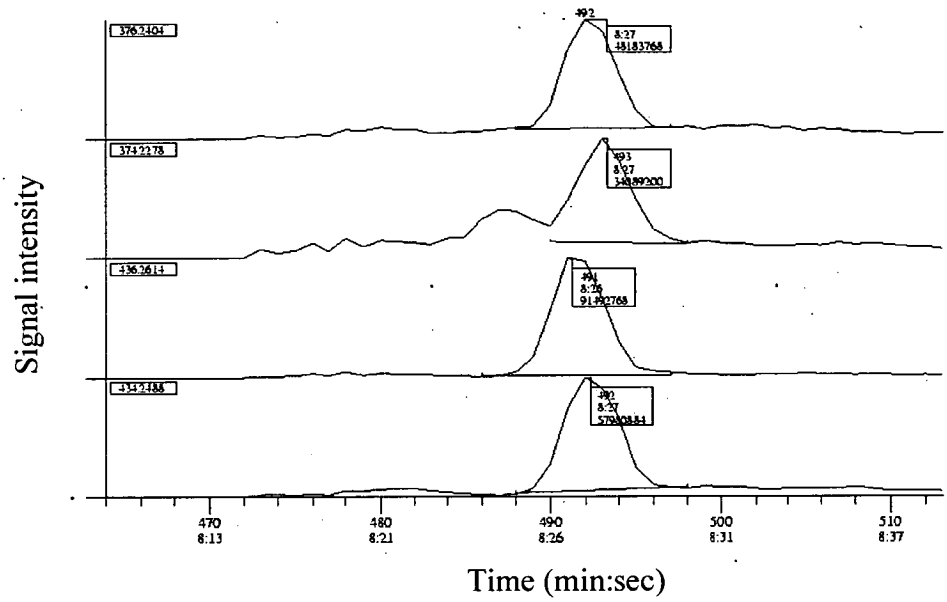


Figure 3.

Typical mass chromatograms of derivatised GA_{19}. Masses 434 (endogenous) and 436 (deuterated) were used to determine the levels of GA_{19} from runs on this system, while masses 374 (endogenous) and 376 (deuterated) provide confirmation of the identity and confirm the ratio is not distorted by impurities.

GC-MS-MS

GC-MS-MS was performed on a Varian 3800 gas chromatograph coupled to a Varian 1200 triple quadrupole mass spectrometer controlled by Varian Star software. The column was a Varian 'Factor Four' VF-5ms (26 m x 0.25 mm internal diameter and 0.25 micron film. Varian Inc., Palo Alto, CA., USA), using Varian 1177 split/splitless injector in splitless mode. Ion Source 220°C, transfer line 290°C. The typical injection volume was 1 microlitre in splitless mode. The injector was held at 250°C. The programs used for each hormone analyzed on this system are as follows:

IAA

GC-MS Selected Reaction Monitoring.
Endogenous; *m/z* 261 → 202 (-14 V collision energy).
Labeled; *m/z* 267 → 208 (-14 V collision energy).

Parent isolation 2 amu, daughter 1 amu. Cycle time 0.3 seconds.
Column oven 50°C for 2 minutes then to 190°C at 30°C/min, then to 220°C at 10°C/minute, then to 270°C at 30°C/minute.
GC carrier flow was helium at 1.4 mL/minute (constant flow mode).
High gain on detector, retention time ~ 8.8 minutes.

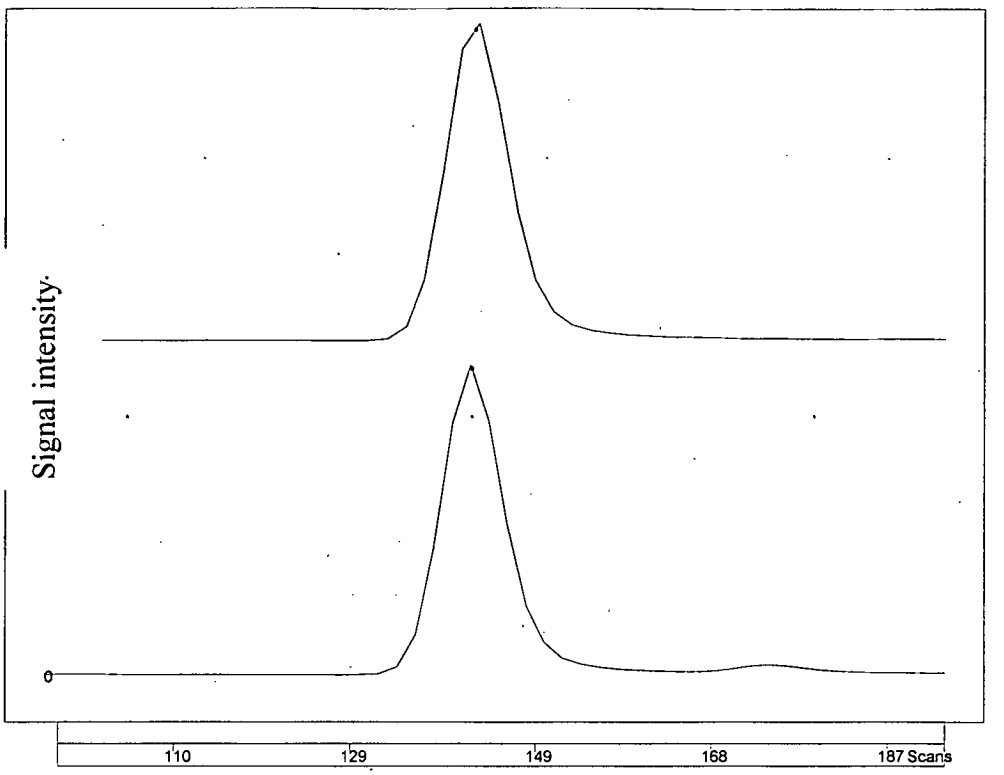


Figure 4.

Typical mass chromatograms of derivatised IAA. Endogenous IAA upper, [^{13}C]IAA lower. There is no offset as the [^{13}C] form has an identical GC retention time to the endogenous form. There were no significant difficulties quantifying IAA in any of the experiments.

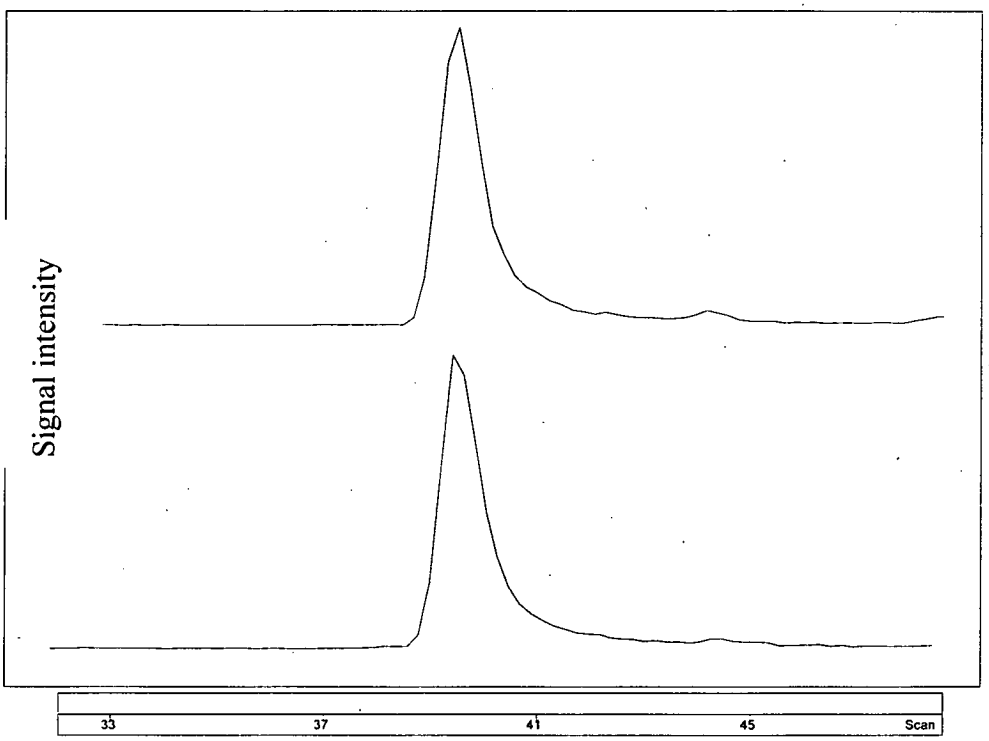
2,4-D

GC-MS Selected Reaction Monitoring.

Endogenous; *m/z* 234 →198 to 202 (to collect Cl isotopes at 199 & 201) (-8V collision energy).

Labeled; *m/z* 240 → 204 to 208 (to collect Cl isotopes at 205 & 207) (-8V collision energy).

Parent isolation 5 amu, daughter 1, 2 amu. Cycle time 0.3 seconds. Column oven and flow as for IAA. High gain on detector, retention time ~ 7.4 minutes.



Time (decimal minutes)

Figure 5.

Typical mass chromatograms of derivatised 2,4-D. Unlabelled (applied) 2,4-D, upper; [^{13}C] labeled internal standard, lower. The "tailing" of the peaks is typical of methyl-ester derivatives.

GA_{20}

GC-MS Selected Reaction Monitoring.

Endogenous; *m/z* 418 → 375 (-12V collision energy).

Labeled; *m/z* 420 → 377 (-12V collision energy).

Parent isolation 1.5 amu, daughter 1.8 amu. Cycle time 0.3 seconds. Column oven 50°C for 2 minutes then to 230°C at 30°C per min then to 270°C at 5°C per minute then hold 2 minutes. GC carrier flow was helium at 1.2 mL minute (constant flow mode). High gain on detector, retention time ~ 12.5 minutes.

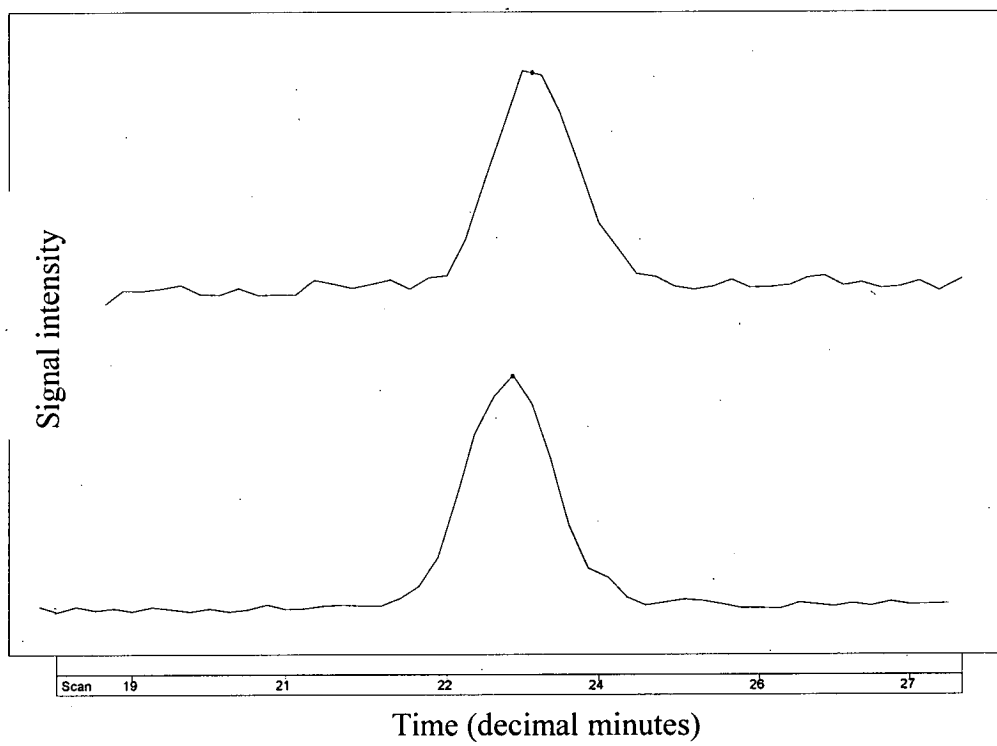


Figure 6.

Typical mass chromatograms of derivatised GA_{20}. Endogenous GA_{20}, upper; $[^2H]GA_{20}$, lower. The dual-MS procedure was a substantial improvement for some aliquots of this GA compared with high resolution GC-MS.

GA_{19}

GC-MS Selected Ion Monitoring.
Endogenous; *m/z* 374 and 434.
Labeled; *m/z* 376 and 436.

Isolation 1.5 amu, Cycle time 0.3 seconds. Column oven 50°C for 2 minutes then to 230°C at 30°C per min then to 270 at 5°C per minute then hold 2 minutes. High gain on detector, retention time ~ 13.4 minutes. Quantitation based on the sum of the target ions for each compound.

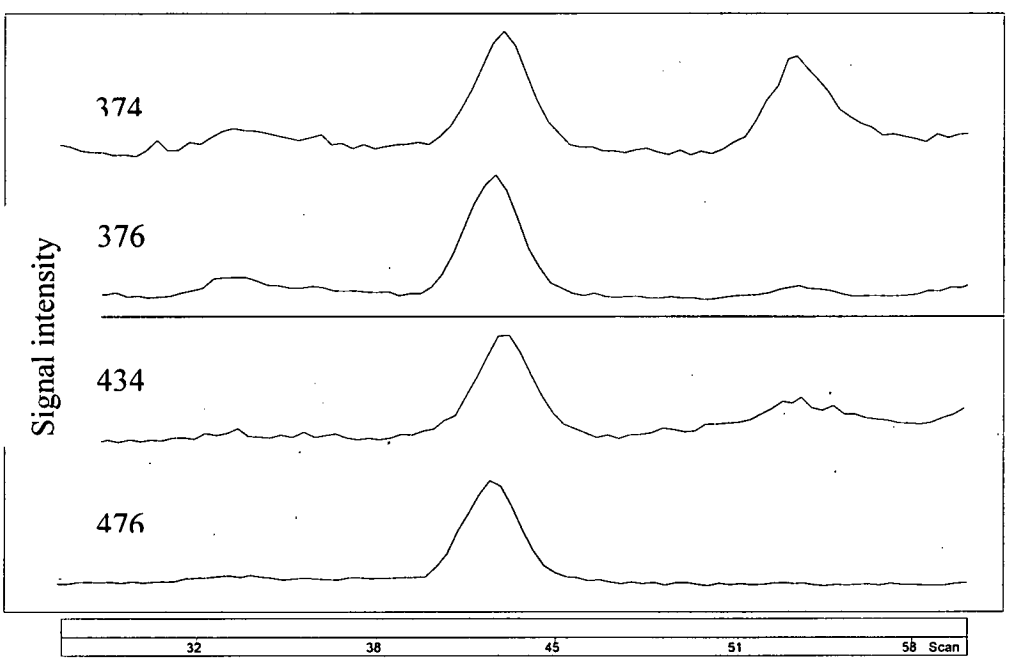


Figure 7.
Typical GC-MS mass chromatograms of GA_{19}. Two mass pairs were monitored for GA_{19} on this system, 374/376 shown above and 434/436 shown below (endogenous/deuterated respectively). GA_{19} was run successfully on either GC-MS system and there was no detectable difference in run quality between the two systems.

GC-MS Analysis

Correction factors were applied to all GA runs to compensate for both isotopic contributions to the labelled peak area from endogenous material and un-labelled hormone present in the deuterated standards. These were as follows:

For Kratos GC-MS runs:

GA_1 corrected 506 = total 506 – (0.004 x total 508)

corrected 508 = total 508 – (0.10 x corrected 506)

GA_8 corrected 594 = total 594 – (0.023 x total 596)

corrected 596 = total 596 – (0.087 x total 594)

GA_{29} corrected 506 = total 506 – 0.005 x total 508

corrected 508 = total 508 – 0.10 x corrected 506

For Varian GC-MS-MS

GA_{20} corrected 418 = total 418 – (0.007 x total 420)

corrected 420 = total 420 – (0.13 x corrected 418)

GA_{19} corrected 434 = total 434 – 0.115 x total 436

corrected 436 = total 436 – 0.29 x corrected 434

No correction factor was applied to IAA or 2,4-D as there was no "cross-talk" between the standard and the endogenous channels. Hormone content was calculated using the formula

$$\frac{\text{Corrected endogenous peak area}}{\text{Corrected internal standard peak area}} \times \frac{\text{Internal standard (ng)}}{\text{Fresh weight (g)}}$$

Technical Problems

Of the hormones analysed on the Kratos GC-MS system, all but GA_1 were run with reasonable success on the HP1 column. All GA_1 samples from both seedling stems and vascular cambium were found to contain large quantities of an impurity that co-eluted with GA_1 on the HP1 GC column and produced a large 507 amu fragment (Figure 12) that obscured the deuterated GA_1 508 channel via isotope effect. Manipulation of the extraction and clean-up protocols for GA_1 to remove this impurity was one of the most time consuming aspects of this project.

The 507 amu impurity also co-eluted with GA_1 as a free acid on HPLC. The addition of the methyl-ester HPLC step was found to lower the content of this impurity significantly but large amounts were still present. GA_1 was also obscured at times by a 504 amu impurity (Figure 13), which impacted on the runs in a similar manner to the 507 amu impurity.

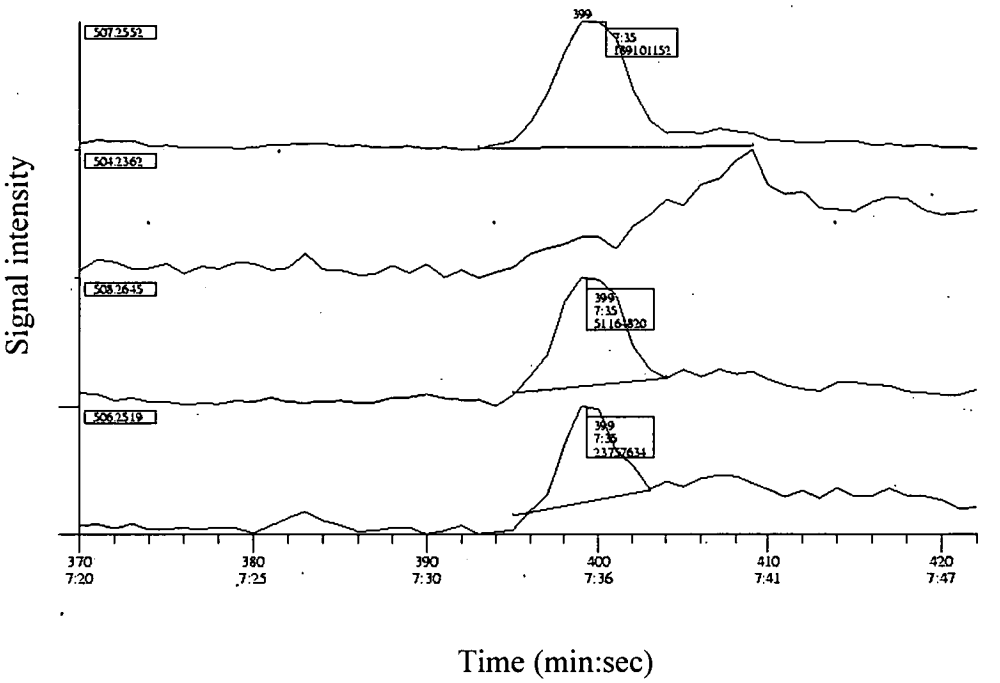


Figure 8.

Mass chromatograms from a high resolution GA_1 GC-MS run showing a large amount of 507 amu impurity co-eluting with GA_1 on the HP1 column. Masses shown (top to bottom) 507.2552, 504.2362, 508.2645 (deuterated GA_1 mass) and 506.2519 (endogenous GA_1 mass). This impurity frequently co-eluted with GA_1 on the HP1 column. The potential for this impurity to artificially inflate GA_1 peak areas rendered these runs un-usable.

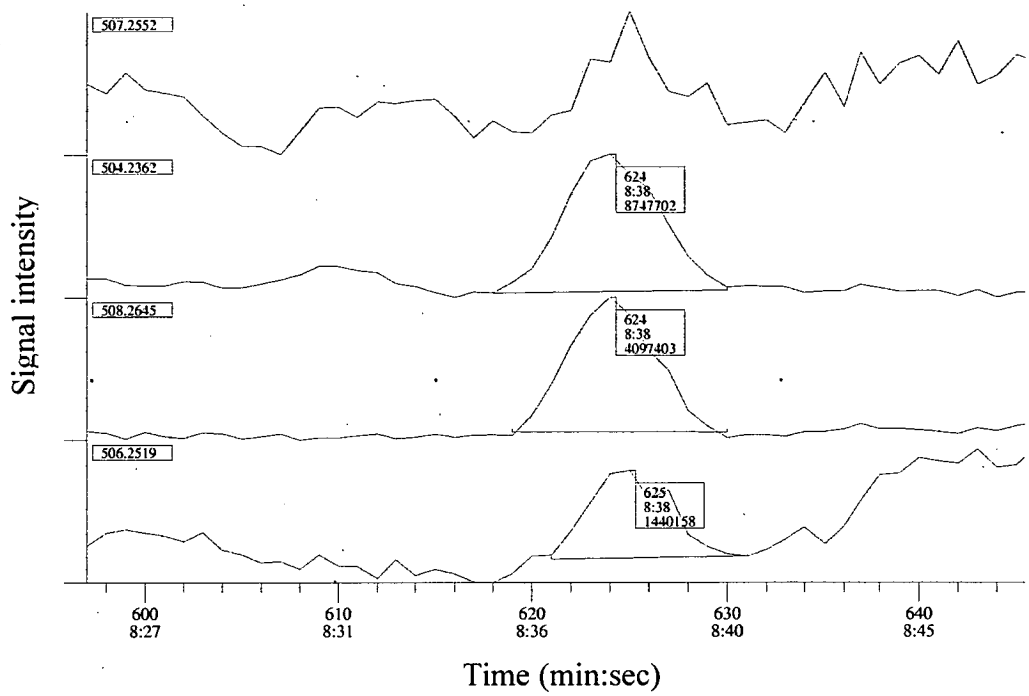


Figure 9.

Mass chromatograms of GA_1 from HP1 column contaminated with the 504 amu impurity. Masses shown (top to bottom) 507.2552, 504.2362, 508.2645 (deuterated GA_1 mass) and 506.2519 (endogenous GA_1 mass). Although the 504 impurity co-eluted with GA_1 infrequently, the potential for this compound to artificially inflate GA_1 peak areas renders these runs un-usable.

Sep Pak-anion exchange

Separation with either Acell™ Plus QMA (strong anion-exchanger) or Oasis® MAX cartridges (reversed phase and strong anion–exchanger, both Waters Assoc. Milford, MA USA.) was also trialled as Hasan *et al.* (1994) reported improved HPLC resolution when an anion–exchange step was performed. Although GAs were easily eluted from these products and substantial (visible) impurities remained behind, they did not separate the 507 impurity from GA_1.

Higher mass resolution (~7000) did reduce the signal from this impurity, but not sufficiently to allow quantification of GA_1. The impurity was also found to be remarkably "sticky" in the GC system, "ghosting" onto 10 or more subsequent blank runs with only slightly diminished signal strength on each successive run. When multiple GA_1 runs were attempted, the impurity built up very quickly in the system and was very time-consuming to remove.

In later experiments the use of a BPX-608 GC column allowed separation of this impurity and GA_1 (Figure 14), but GA_1 levels were found to generally be below detectable levels. Full scan GC-MS revealed a molecular mass of 566 amu for the impurity.

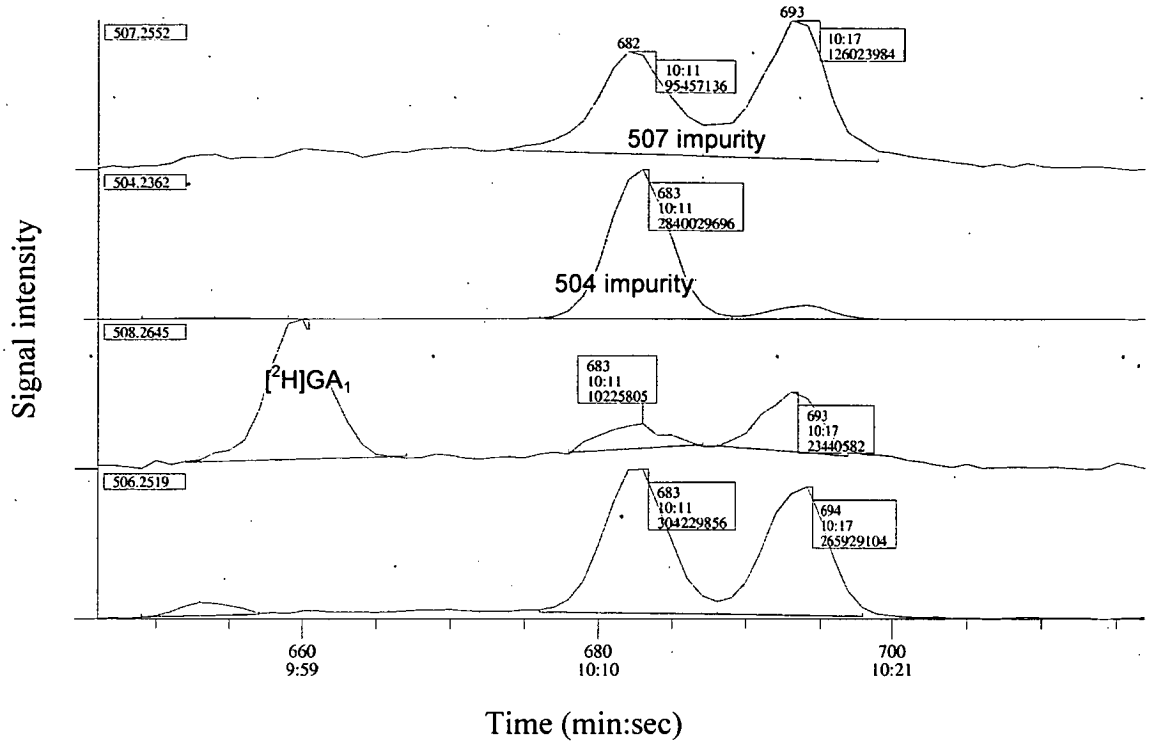


Figure 10.

Mass chromatograms of a GA_1 GC-MS run with the BPX-608 GC column. Masses shown (top to bottom) 507.2552, 504.2362, 508.2645 (deuterated GA_1 mass) and 506.2519 (endogenous GA_1 mass). Both 507 and 504 amu impurities have been separated from GA_1. However, levels of endogenous (506 amu) GA_1 are too low for quantification.

Towards the end of the project, diazomethane became unavailable and (trimethylsilyl) diazomethane was used as a methylating agent. Whilst this agent was as effective as diazomethane, it was found to contain impurities that co-elute with GA on GC-MS. Dilution of (trimethylsilyl) diazomethane 1:10 with di-ethyl ether reduced the levels of impurity sufficiently to allow GA quantification but a number of samples were contaminated before this was discovered.

Chapter 3 - Auxin and gibberellins in seedlings

Introduction

The shoot tip has been well established as an important source of IAA found in the stems of plants. Decapitation typically causes a substantial reduction in stem IAA content and this treatment has long been used to study the effects of basipetally transported IAA in the stems of plants. Defoliation has also recently been shown to reduce IAA content in some experimental systems. In pea, Cambridge and Morris (1996) suggested that IAA applied to mature leaves is loaded into the phloem and then into the basipetal IAA stream. Further work by Jager *et al.* (2007) has demonstrated de-novo IAA biosynthesis in mature leaves of pea as well as a reduction in stem IAA content when plants were defoliated. Experiments with herbaceous species have shown that decapitation reduces bioactive GA levels, and that application of IAA partially or completely restores GA levels (discussed above).

Very similar results to those from herbaceous species have been observed in trees when stems are decapitated and/or defoliated (Wang *et al.* 1997; McElwee 2003; Bjorklund *et al.* 2007). The effects of altered hormone levels on the vascular cambium and its derivatives has also been studied. In *P. sylvestris* seedlings decapitation has been shown to reduce IAA content as well as both xylem and phloem production in the stem but there was no reduction in stem elongation or GA content. Defoliation was found to reduce both IAA and GAs in the stem, as well as shoot elongation and cambial growth (Wang *et al.* 1997).

Previous work (McElwee 2003) has indicated that both leaves and the stem apex are important sources of IAA in the elongating stems of *E. globulus* seedlings and that IAA may influence GA levels in seedling stems. The dominant pathway for the synthesis of bioactive GA in *E. globulus* is the early 13-hydroxylation pathway, where GA_{12} is converted to $GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20}$ (Hasan *et al.* 1994; Ridoutt and Pharis 1998).

To further investigate the possibility of an interaction between IAA and GA levels in *E. globulus* seedling stems, levels of GA_{19}, GA_{20}, GA_1, GA_{29} and GA_8 were monitored across intact, decapitated and decapitated +IAA treatments. This experimental system was designed to closely resemble experimental systems used in herbaceous species to investigate IAA/GA interactions. The aim of these experiments was to investigate the possibility of a similar interaction between IAA and GA in *E. globulus* seedlings and to collect data from the primary growth of *E. globulus* to enable comparison with secondary growth from this species. Due to difficulty obtaining accurate GA_1 levels from the seedling stems, this experiment was repeated with revised methods. However, GA_1 levels remained elusive and below detectable levels in most aliquots.

Methods

Growth Conditions

For the earlier seedling experiments, plants were sourced from the Forestry Tasmania nursery in Perth, Tasmania and acclimated to greenhouse conditions (detailed below) for 2 months before experiments began. For later seedling experiments, plants were germinated from seed (size 1.7 mm or greater) in trays on the soil described below with a 10 mm covering layer of vermiculite. Seedlings were germinated under 24 hour lighting at 23°C. Germinated seedlings were transferred to individual pots 5 cm in diameter after 8 days.

After potting, seedlings were grown in a greenhouse with temperatures ranging from 16-24°C (summer) to 10-18°C (winter) under natural daylight averaging 14-16 mol^{-2} d^{-1}. Once seedlings reached approximately 30 cm in height they were transferred to pots of 10 cm diameter. Soil was a mix of 6 parts composted fine pine bark, 4 parts coarse washed river sand and 1 part moss. The pH was adjusted to around 6 by adding dolomite lime at 2.7 kg/m^3. Nutrient was supplied by 3-4 month Osmocote® N:P:K 19:2.6:10 added at 1 kg/m^3, 8-9 month Osmocote® N:P:K 17:1.6:8.7 (2 kg/m^3), Micromax® (0.5 kg/m^3) and ferrous sulphate (0.5 kg/m^3) (Osmocote® and Micromax®, Scotts-Sierra Horticultural Products Co. USA).

Treatments

Seedlings were treated by removal of the apical portion (all tissue above the uppermost node with leaves greater than 5 mm in length) and leaves from the 3 nodes below this. Any lateral shoots or visible buds were also removed from this zone (Figure 11). Previous experimentation (McElwee 2003) indicated that typically all of these internodes are elongating in seedlings of

E. globulus of this age. Lanolin containing either IAA (7.5 mg/g) and 600 µL ethanol or ethanol only was applied to the cut stump of the apex immediately after decapitation/defoliation and approximately every 12 hours for 48 hours. Harvested tissues consisted of the internodes above the highest remaining leaf-bearing node in the case of decapitated/defoliated seedlings or equivalent tissue in intact plants. Each stem tip, contaminated with exogenous IAA/lanolin mixture or lanolin only, was excised and not included in the tissue analysed. Stem segments were weighed and placed in cold (-20°C) 80 % (v/v) MeOH containing 250 mg BHT/L. Subsequent purification and analysis was carried out as described in chapter 2.

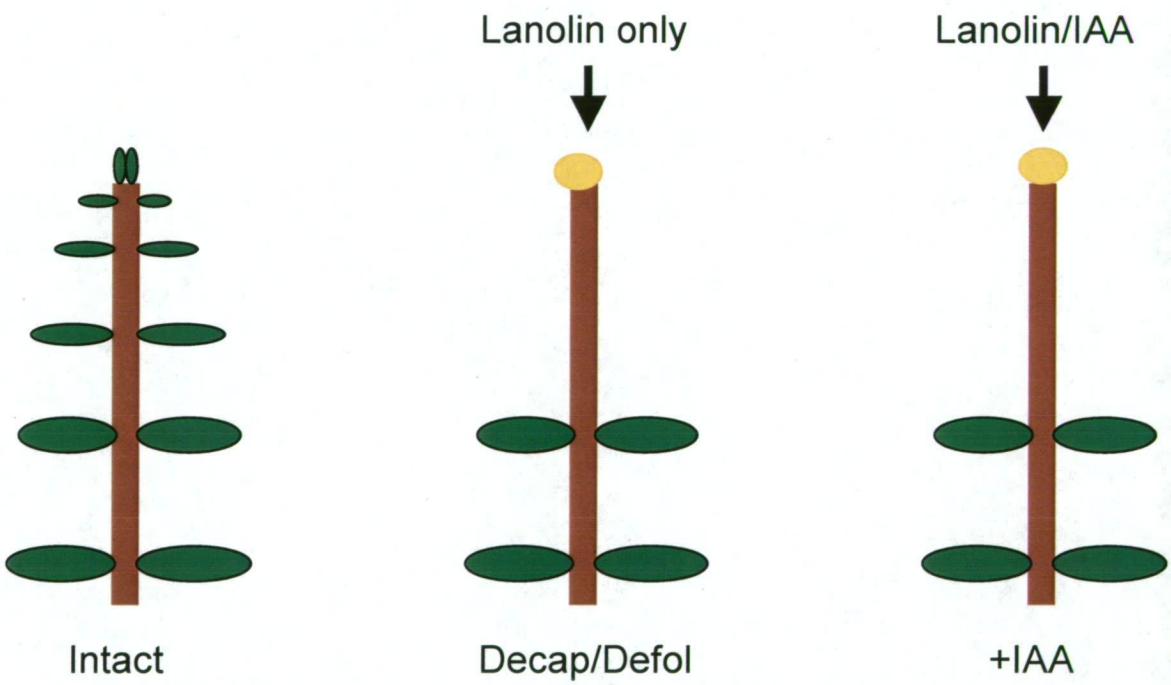


Figure 11.
Experimental treatments applied to seedlings of *E. globulus*. IAA was dissolved in 600 µL of ethanol and applied in lanolin at 7.5 mg/g. Decapitation/Defoliation consisted of removal of all tissue above the uppermost node that was bearing leaves greater than 5 mm in length, as well as leaves from the 3 nodes below this node.

Results

In spite of previous success with new methods of extracting and quantifying GA_1 from *Eucalyptus* seedling stems in pilot experiments, this success was not reproducible in these experiments. Metabolites that interfere with GC-MS quantification of GA_1 were not consistent across experiments. Each batch of experimental tissue contained a different set of impurities and methods of separating GAs from interfering compounds on GC-MS had to be refined for each seedling experiment.

The first experiment consisted of 3 replicates each of intact, decapitated and decapitated +IAA treatments. From this experiment levels of IAA, GA_{20} and GA_8 were obtained. This experiment was conducted before the BPX-608 gas chromatograph column was trialled and levels of GA_1 were not able to be quantified.

Levels of IAA were reduced approximately 8-fold by decapitation/defoliation treatment and elevated to similar levels as intact plants by application of IAA to the cut stump in lanolin paste. The statistical significance of the recovery of auxin levels by IAA application (t-test, $P=0.053$) was reduced by the large standard error in these data, caused by one +IAA replicate having twice as much IAA as intact plants.

On average GA_{20} was reduced to 6-fold less than levels in intact plants and restored to just less than half intact levels by IAA application (Figure 12). GA_8 also responded to both decapitation/defoliation and IAA application but to a lesser degree than GA_{20}. Levels of GA_8 were reduced approximately 2-fold by decapitation/defoliation and restored to approximately 80 % of intact levels by IAA application (Figure 12). All of these changes in GA levels were statistically significant (t-test, $P<0.05$) (Table 2).

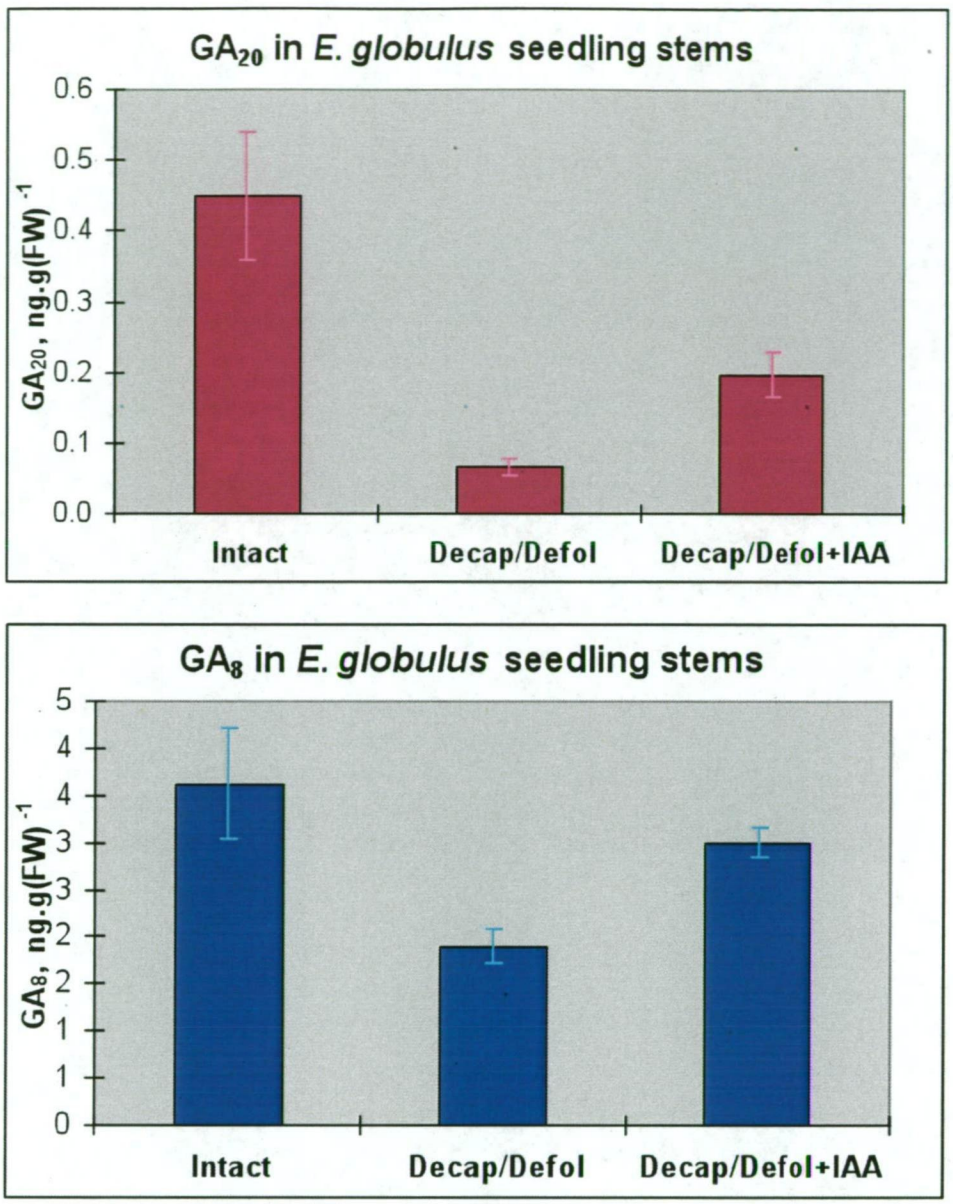


Figure 12.

Effect of decapitation/defoliation and IAA application on levels of GA_{20} and GA_8 in elongating *E. globulus* seedling stems. Seedlings were either left intact until harvest or decapitated and defoliated 48 hours before harvest. Where IAA was applied, it was dissolved in 600 µL ethanol and mixed with lanolin paste at 7.5 mg/g. Ethanol/lanolin mixture was applied to decap/defol plants. Re-application was every 12 hours over a total treatment time of 48 hours. Bars indicate standard error and n=3.

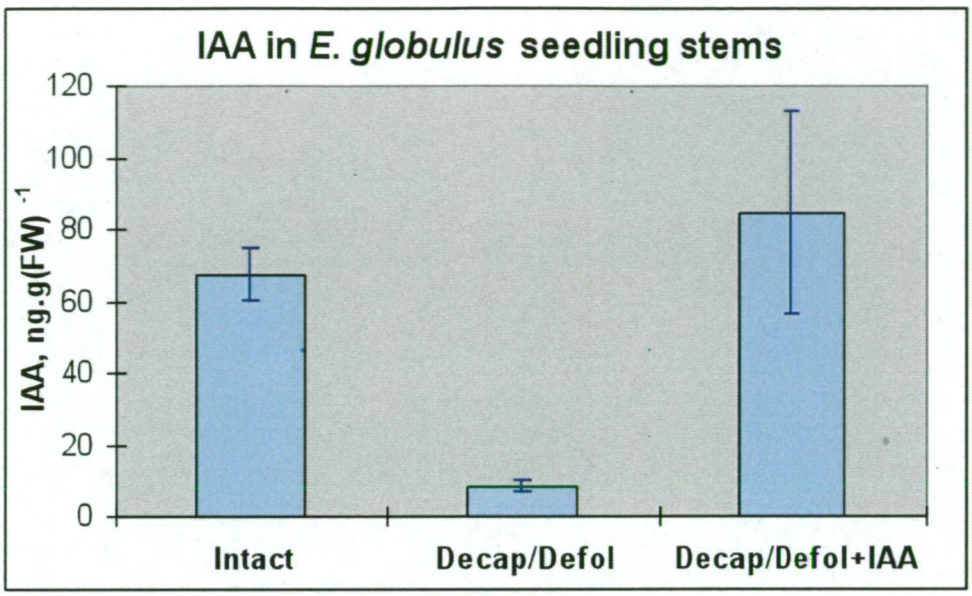


Figure 13.

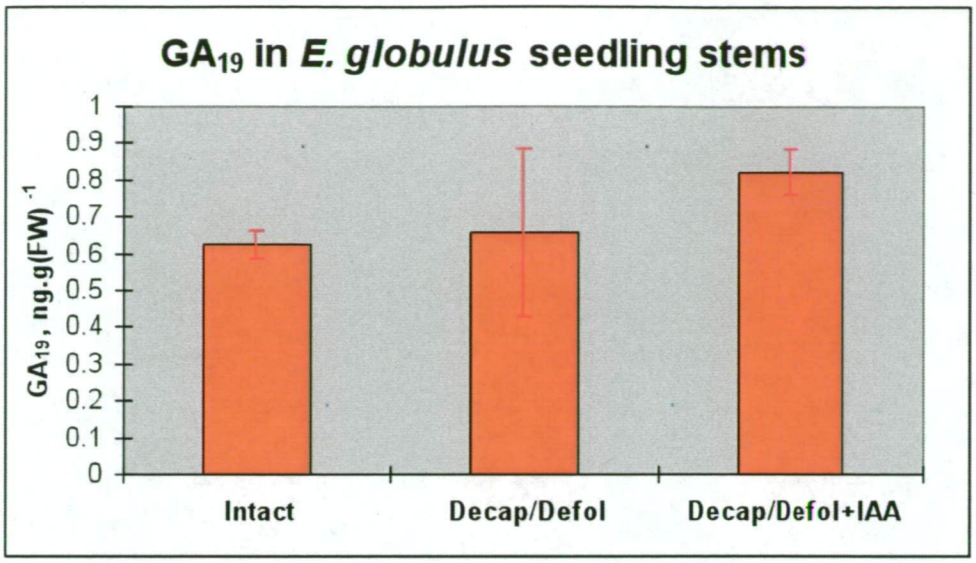
Effect of decapitation/defoliation and IAA application on levels of IAA in elongating *E. globulus* seedling stems. Seedlings were either left intact until harvest or decapitated and defoliated 48 hours before harvest. Where IAA was applied, it was dissolved in 600 µL ethanol and mixed with lanolin paste at 7.5 mg/g. Ethanol/lanolin mixture was applied to decap/defol plants. Re-application was every 12 hours over a total treatment time of 48 hours. Bars indicate standard error and n=3.

Table 2.

Student's t-test of the data from Figure 13, decapitation/defoliation significantly ($P \leq 0.05$) reduced all hormones quantified relative to intact plants, while application of IAA significantly increased gibberellins relative to Decap/Defol plants.

P	GA_{20}	GA_8	IAA
Intact/DecapDefol	0.0113	0.0492	0.0014
Intact/+IAA	0.0485	0.3622	0.5843
DecapDefol/+IAA	0.0179	0.0104	0.0532

The second repetition of this experiment contained 2 repetitions of intact plants and 3 repetitions of decapitated/defoliated and decapitated/defoliated + IAA treated plants. Levels of IAA and GA_{19} (Figure 14 and Table 3) were obtained along with an incomplete set of GA_8 and GA_{20} (not shown) results. Use of the BPX-608 gas chromatograph column allowed separation of GA_1 from the 507 and other impurities but GA_1 was below detectable levels.



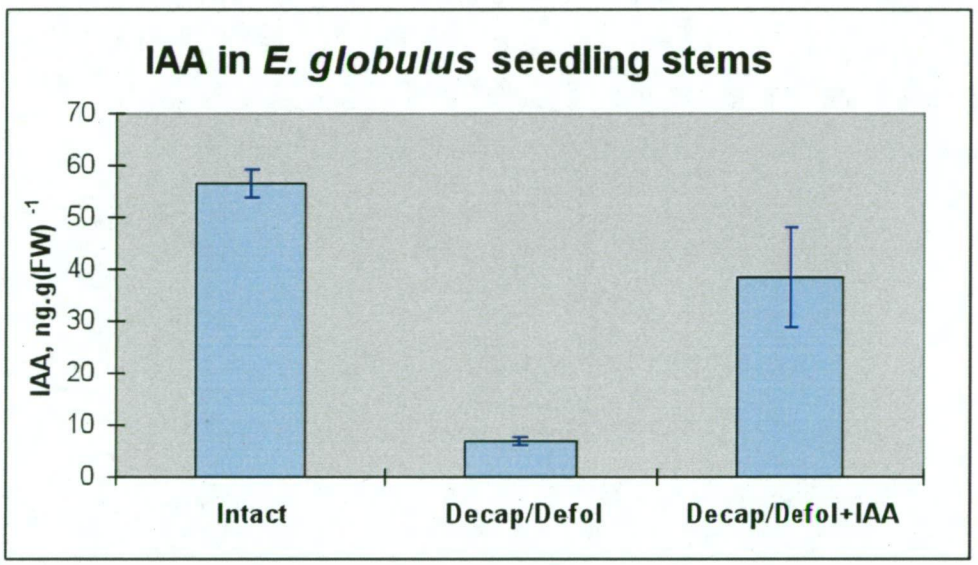


Figure 14.

Effect of decapitation/defoliation and IAA application on levels of IAA in elongating *E. globulus* seedling stems. Seedlings were either left intact until harvest or decapitated and defoliated 48 hours before harvest. Where IAA was applied, it was dissolved in 600 µL ethanol and mixed with lanolin paste at 7.5 mg/g. Ethanol/lanolin mixture was applied to decap/defol plants. Re-application was every 12 hours over a total treatment time of 48 hours. Bars indicate standard error, n=2 for intact plants and n=3 for Decap/Defol and Decap/Defol+IAA.

Table 3.

t-test of the above data, decapitation significantly ($P<0.05$) reduced IAA relative to intact plants, while application of IAA did not significantly impact upon GA_{19} levels.

P	IAA	GA_{19}
Intact/Decap	0.0004	0.9135
Intact/+IAA	0.2479	0.1111
Decap/+IAA	0.0310	0.5312

IAA levels were reduced approximately 10-fold in the second experiment but only restored to $2/3^{rds}$ of intact levels by IAA application. Levels of GA_{19} were not affected by either decapitation/defoliation or IAA application. Levels of GA_{20} were very low in both decapitated/defoliated and decapitated/defoliated +IAA plants and accurate quantification was not possible. Attempts were also made to quantify GA_8 levels from this experiment but impurities prevented accurate quantification of all samples and the results are not included.

Discussion

IAA

IAA levels were consistently and substantially reduced by decapitation/defoliation treatment and considerably restored by IAA application. These changes in IAA levels are comparable to those observed in pea (Ross *et al.* 2000), barley (Wolbang *et al.* 2004), *P. sylvestris* (Wang *et al.* 1997) and hybrid aspen (Bjorklund *et al.* 2007) when similar treatments are applied. The combined decapitation/defoliation treatment used in these experiments was shown to be more effective than either decapitation or defoliation alone in *E. globulus* seedlings (McElwee 2003).

Wang *et al.* (1997) also reported a greater drop in both IAA and GAs when stems of *P. sylvestris* were defoliated or decapitated and defoliated compared to decapitation alone.

Gibberellins

Levels of GA_{19} and GA_{20} have not previously been studied in trees in response to depletion/decapitation and IAA application. The lack of effect of decapitation/defoliation treatment or IAA application on GA_{19} levels observed in these experiments is consistent with results from pea and barley. It is likely that the biosynthetic steps up until GA_{19} are not affected by decapitation or IAA application in these species. It is curious that only the penultimate GA 20-oxidation reaction is affected by decapitation/defoliation or IAA application in these species when GA 20-oxidase is known to be multifunctional and capable of catalysing all of the 20-oxidation biosynthetic steps from GA_{12} or GA_{53} to GA_9 and GA_{20} respectively (Hedden and Kamiya 1997). Conversely, in tobacco GA_{19} levels were reduced (approximately 2-fold) by decapitation and restored by IAA application (Wolbang *et al.* 2004), although this change was of much smaller magnitude than the changes in GA_{20} and GA_1 levels in this species.

In hybrid aspen GA 20-oxidation has been suggested to be the rate limiting step in the biosynthesis of active GAs and its over-expression increased bioactive GA levels more than 20-fold (Eriksson *et al.* 2000). However, up-regulation of *GA20ox1* and *GA20ox2* in decapitated seedlings to which IAA was applied did not result in any increase in GA_1 and a more modest increase in GA_4 of approximately 4-fold (Bjorklund *et al.* 2007).

The reduction of GA_{20} levels observed in these experiments in decapitated/defoliated seedlings of *E. globulus* is similar to the results from tobacco where decapitation reduced GA_{20} by 5-fold and GA_1 20-fold (Wolbang and Ross 2001). However, in pea where GA 3-oxidation is the affected step GA_{20} levels were relatively unaffected by either decapitation or IAA treatment (Ross *et al.* 2000; O'Neill and Ross 2002). Application of IAA to decapitated tobacco plants increased levels of GA_{20} to levels 3-fold higher than intact plants while GA_1 levels were restored to within 70 % of intact levels (Wolbang and Ross 2001). Whilst the changes in GA_{20} levels were of smaller magnitude in *E. globulus* than tobacco, the overall effect of IAA application on GA_{20} levels was the same between these species.

When GA_{20} levels are reduced by decapitation treatment in tobacco, GA_1 levels were reduced by a greater amount (Wolbang and Ross 2001). When GA biosynthesis inhibitors were applied to seedlings of *E. nitens*, the magnitude of reduction of GA_{20} and GA_1 were also similar, regardless of the particular biosynthetic step inhibited (Williams *et al.* 1999). It is therefore thought likely that the reductions in GA_{20} observed in these experiments would have also resulted in a reduction of GA_1 levels in decapitated/defoliated plants, and that a raised level of GA_{20} following application of IAA is indicative of an increase in GA_1 levels of a similar order.

Levels of the GA_1 metabolite GA_8 were also reduced by decapitation/defoliation treatment and were either elevated or completely restored by IAA application. A reduction in GA_8 levels and recovery by IAA application of similar magnitude was reported by Ross *et al.* (2000) in pea while in barley neither decapitation or IAA application had any substantial effect on levels of GA_8 in nodes or internodes (Wolbang *et al.* 2004).

In hybrid aspen, GA_{34} (the deactivation product of GA_4) was elevated approximately 2-fold by IAA application compared to decapitated seedlings (Bjorklund *et al.* 2007).

The lack of effect of decapitation/defoliation on the levels of GA_{19} suggests that in the elongating stems of *E. globulus* GAs are synthesised *in situ* rather than from a precursor transported from the apical bud or leaves. This is supported by, although not directly comparable to, the findings of Isrealsson *et al.* (2005) that the later stages of GA biosynthesis occur in the vascular cambial region in 40-year-old *Populus tremula* during active growth.

Although GA_1 was not quantified in these experiments, the internal standard (1ng $[^2H]GA_1$) was easily detected on GCMS suggesting that GA_1 levels were at least 10 fold lower than this. The low concentration of GA_1 in *E. globulus* in these experiments is contrary to previous work on this species where levels reported were generally much higher (Hasan *et al.* 1994; Ridoutt *et al.* 1995; 1996; Matyczek 1995; Williams *et al.* 1999). However, the tissues analysed in these experiments differ from those in previous experiments (leaves were not included). It was expected that elongating stems would contain similar, if not greater, levels of bioactive GA than combined leaf and stem extracts given the strong links between GAs and cell elongation but this does not seem to be the case.

The methods used in these experiments differ from those of Hasan *et al.* (1994) in some ways. Samples harvested by Hasan *et al.* (1994) were placed in a freezer at -15°C before being harvested and ground in a mortar with liquid nitrogen, while in these experiments samples were transported 2 hours at room temperature until being harvested and placed in cold 80 % methanol with BHT. Hasan et al (1994) partitioned against ethyl acetate very early in the procedure whereas in these experiments the samples were partitioned

against di-ethyl ether after free acid HPLC and methylation but before the second methyl-ester HPLC run (if a methyl-ester HPLC run was performed). Hasan *et al.* (1994) also utilised an additional separation step with the QAE Sephadex A25 which was not used in these experiments. This step was added by these authors to address the problem of very broad HPLC retention times, a problem not encountered in these experiments due to the much lower amounts of sample available (typically less than 5g in these experiments compared to 100g or more).

The additional methyl-ester HPLC step was not used by Hasan *et al.* (1994) and the protocol for the use of C18 Sep-Pak cartridges in this project is a refinement on that used by these authors. It is not thought likely that either of these differences in procedure would have impacted on the quality of the results obtained and that the much lower amounts of sample available were responsible for the more variable success of the procedures used in these experiments. All other differences between the protocols used are additions or refinements to the procedure and it is not thought likely that they would have negatively impacted on the quality of the results.

Overall, the effects of decapitation/defoliation treatment and IAA application in *E. globulus* seedlings are very similar to those observed in other species. These effects of IAA on the GA biosynthetic pathway have not previously been demonstrated in eucalyptus. The response of GA_{20} and GA_8 levels to IAA application suggests that IAA does have a role in promoting the biosynthesis of GAs in this system and although GA_1 was not directly quantified, it is likely that its levels are reduced by treatment and may be somewhat restored by IAA application.

Chapter 4– Gibberellin metabolism in excised seedling stems.

Introduction

The metabolism of radio-labelled GAs has previously been used to examine both GA biosynthesis pathways and the effects of IAA on these pathways. Radio-labelled GAs have been added to a variety of tissues and experimental systems and have been shown to be rapidly transported and metabolised both in whole plants and excised tissues.

In pea internodes, biosynthesis of GA_1 from labelled GA_{20} is inhibited by decapitation and restored by IAA application. Also, deactivation of GA_{20} to GA_{29} was found to be promoted by decapitation and inhibited by application of IAA (Sherriff *et al.* 1994; Ross *et al.* 2000). When pea internodes were excised and floated on MS media the same interaction was observed (Sherriff *et al.* 1994; O'Neill and Ross 2002). Identical results were obtained when these experiments were repeated in both decapitated tobacco (Wolbang and Ross 2001) and barley as well as in excised stems of barley in MS media (Wolbang *et al.* 2004). However, in contrast to herbaceous species, Bjorklund *et al.* (2007) found only a slight increase in GA_4 and no increase in GA_1 when IAA was applied to decapitated hybrid aspen seedlings.

In *P. sylvestris*, deuterated and tritiated GA_9, GA_4 or GA_{20} were applied to the midpoint of the previous year terminal shoot (Wang *et al.* 1996). All GAs were found to be readily transported into all tissues analysed although the highest levels of labelled GA_{20} and GA_9 were retained at the application site. The majority of labelled GA_4 recovered was from the current year terminal shoot. GA_9 was found to be converted to GA_{20}, GA_4 and GA_1 and applied GA_4 was found to be metabolised to GA_1. When GA_{20} was applied the sole

metabolite was the inactive GA_{29} (Wang *et al.* 1996). When labelled GAs were applied to the stems of *E. globulus* saplings or seedlings, GA_{20} was converted to GA_1 and GA_{29}, labelled GA_1 was converted to GA_8, GA_4 converted to GA_{34} and GA_9 converted to GA_{51} (Ridoutt and Pharis 1998).

The difference in the metabolism of GA_{20} between these two species is likely due to the probable difference in GA biosynthesis pathways that are predominant in these two very different species. The early 13-hydroxylation pathway ($GA_{19}\rightarrow GA_{20}\rightarrow GA_1$) has been suggested to be the dominant pathway in *E. globulus* (Hasan *et al.* 1994; Ridoutt *et al.* 1995; Ridoutt and Pharis 1998) whereas the dominant biosynthesis pathway in *P. sylvestris* is likely to be via late 13-hydroxylation ($GA_9\rightarrow GA_4\rightarrow GA_1$; Wang *et al.* 1995; 1996; Little and MacDonald 2003).

In the experiments described in the previous chapter it was often not possible to accurately quantify GA_1 due to the difficulty of separating it from impurities by chromatography and/or simply due to the low levels present. The study of the metabolism of labelled hormone and precursors in isolated stem segments with and without IAA present was trialled as a substitute for measuring endogenous levels after IAA-altering treatments.

Methods

Elongating internodes of *E. globulus* seedling were excised from the plant with a razor blade and leaves, visible lateral buds and the shoot tip were all removed. The stems were then immediately placed on liquid MS media (Figure 15). In the initial experiment some stems were decapitated/defoliated 48 hours before placement in MS media (as described in chapter 3), this additional IAA-reducing treatment was found to be unnecessary. All metabolism experiments were incubated at 20°C with a 16 hour day.

Hormones, when added, were in the following quantities; 400,000 dpm [^{14}C]GA_x and 5 μg/ml of IAA. Specific activity of the GA substrates was 55 mCi/m mol for GA_{20} or GA_{19} and 34 mCi/m mol for GA_1. After 24 hours stem segments were washed twice in distilled water (dH_2O) and placed in cold (-20°C) 80 % (v/v) MeOH with 250 mg BHT/L then transferred to a freezer at -20°C overnight then kept at 4°C for 24 hours. Stem segments were then macerated, filtered, methylated, sep-packed and run as methyl-ester derivatives on HPLC as described in chapter 2 but without addition of additional radio-labelled hormone. 4 ml of Ready-Safe Liquid Scintillation counting cocktail (Beckman Instruments Inc. Fullerton CA, USA) was added to each whole HPLC fraction before radio-counting for 5 minutes on a Beckmann LS 6500 (Beckman Instruments Inc. Fullerton CA, USA). Each treatment consisted of 12-15 stems and these were processed as that treatment. Treatments were not replicated in these pilot experiments as the presence or absence of IAA did not alter the metabolism of gibberellins and more rigorous experiments were therefore not performed.

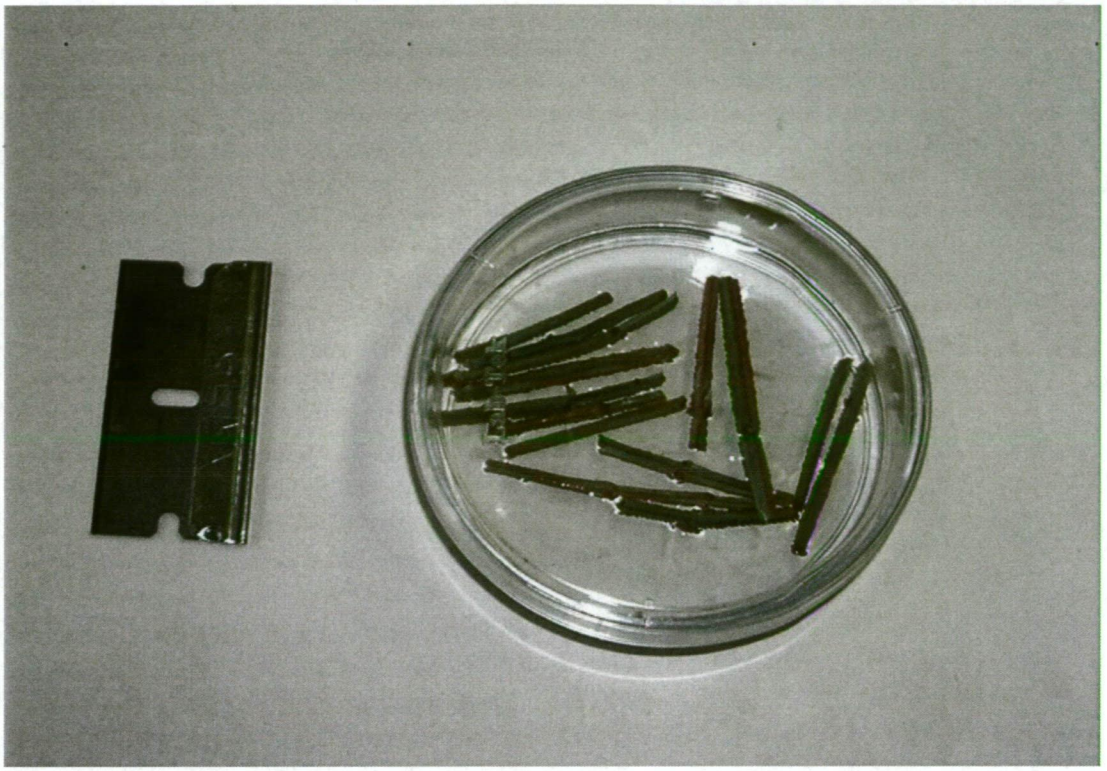


Figure 15.

Excised *E. globulus* seedling stem segments floating on liquid MS media. Radio-labeled gibberellins and IAA were added to the media and GA metabolism in the presence or absence of IAA was studied.

Results and Discussion

There was no significant difference in GA metabolism between stem segments cultured with or without additional IAA for any of the GAs tested.

For the initial experiment where GA_1 metabolism was studied (Figure 16), the level of IAA in stem segments was quantified by GC-MS (Figure 17). Stems incubated in media to which IAA had been added were found to contain approximately 160 ng/gFW^{-1}, about double the level observed in intact seedling stems (see previous chapter). Stems incubated without IAA present in the media contained 3-4 ng/gFW^{-1} of IAA after 24 hours, regardless of decapitation immediately prior to placement in media or 48 hours previously. Additionally, there was no significant difference in GA_1 metabolism between stems that had been decapitated/defoliated 48 hours before harvest and those that had been excised and defoliated immediately prior to being placed in MS media. This suggests that any residual IAA that may have been present in stems immediately after harvesting had no influence on the proportions or levels of labelled substrate and metabolite by the end of the 24 hour period.

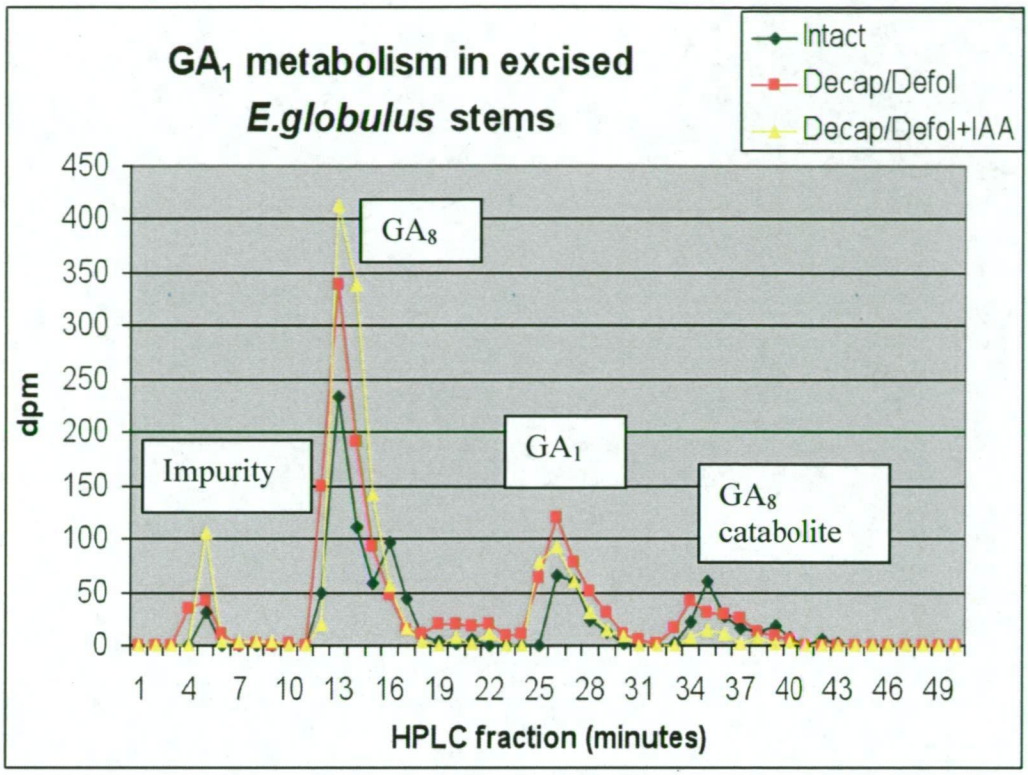


Figure 16.

[^{14}C]GA_1 was fed to excised, elongating *E. globulus* seedling stem segments floating in MS media and its metabolites examined by radio-counting of methyl-ester HPLC fractions. Elongating *E. globulus* seedling stems were excised from the plant and floated in liquid MS media containing 400 000 dpm of [^{14}C]GA_1 (specific activity 34 μCi/μmol) for 24 hours. 5 μg/ml IAA was added to the decap/defol+IAA treatment. 'Decap/Defol' plants were decapitated/defoliated 48 hours before the stem sections were excised to ensure IAA depletion, 'Intact' stem segments were decapitated and defoliated immediately prior to being placed in the MS media. Radioactivity (disintegrations per minute) as measured by liquid scintillation counting of 1 minute HPLC fractions, n=1.

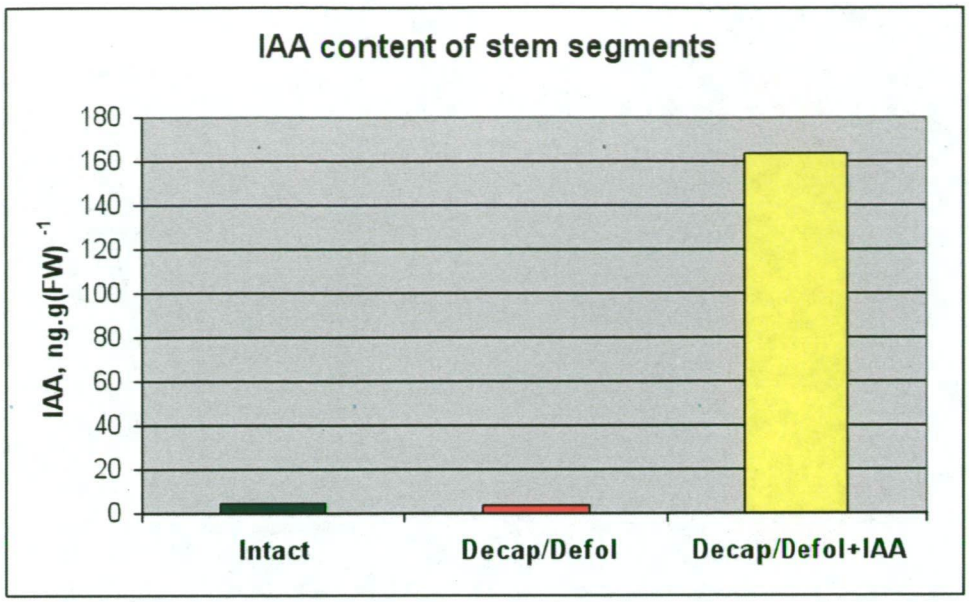


Figure 17.

Levels of IAA in elongating stem segments of *E. globulus* seedlings floated on liquid MS media for 24 hours, n=1. 'Decap/Defol' stems were decapitated and defoliated 48 hours before placement on MS media to ensure IAA depletion while 'Intact' stems were decapitated and defoliated immediately prior to placement on MS media. IAA was added to the MS media at 5 μg/ml. Typical IAA concentration in similar stems freshly harvested elongating stems is 50-70 ng/gFW^{-1}. Prior decapitation/defoliation treatment does not seem to be required to reduce IAA in this experimental system.

Subsequent experiments examined the metabolism of GA_{20} and GA_{19} in excised seedling stems (Figures 19 and 20). The lack of effect of IAA on GA metabolism was though to possibly be due to poor uptake of hormones from the MS media. To test this, an experiment was conducted where stems were split longitudinally with a razor blade immediately prior to placement in MS media, exposing a much larger area of tissue to the media (Figure 18). This treatment had no effect on GA_{20} metabolism.

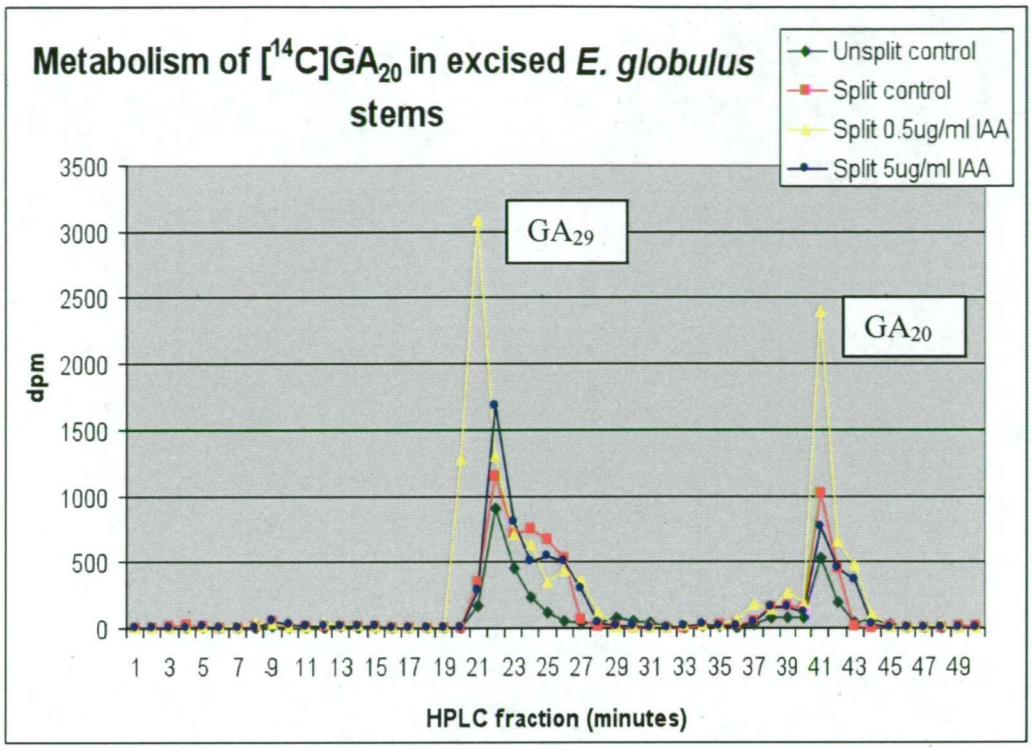


Figure 18.

[^{14}C]GA_{20} was fed to excised, elongating *E. globulus* seedling stem segments floating in MS media and its metabolites examined by radio-counting of methyl-ester HPLC fractions, n=1. Elongating *E. globulus* seedling stems were excised from the plant and floated in liquid MS media containing 400 000 dpm of [^{14}C]GA_{20} (specific activity 55 μCi/μmol) for 24 hours. In the 3 'split' treatments, stems were split longitudinally to better enable uptake of substrate. IAA was added to 2 of the 'split' treatments at either 0.5 μg/ml IAA or 5 μg/ml IAA.

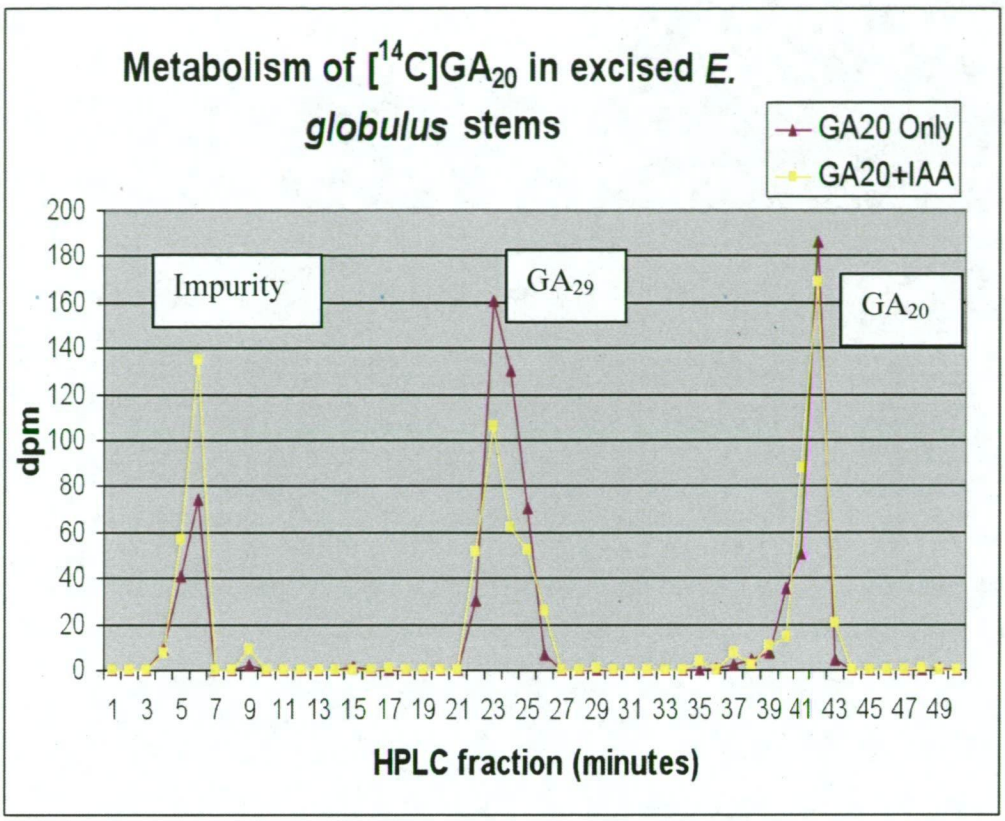


Figure 19.

[^{14}C]GA_{20} was fed to excised, elongating *E. globulus* seedling stem segments floating in MS media and its metabolites examined by radio-counting of methyl-ester HPLC fractions, n=1. Elongating *E. globulus* seedling stems were excised from the plant and floated in liquid MS media containing 400 000 dpm of [^{14}C]GA_{20} (specific activity 55 μCi/μmol) for 24 hours. IAA was added to one of the treatments at 5 μg/ml.

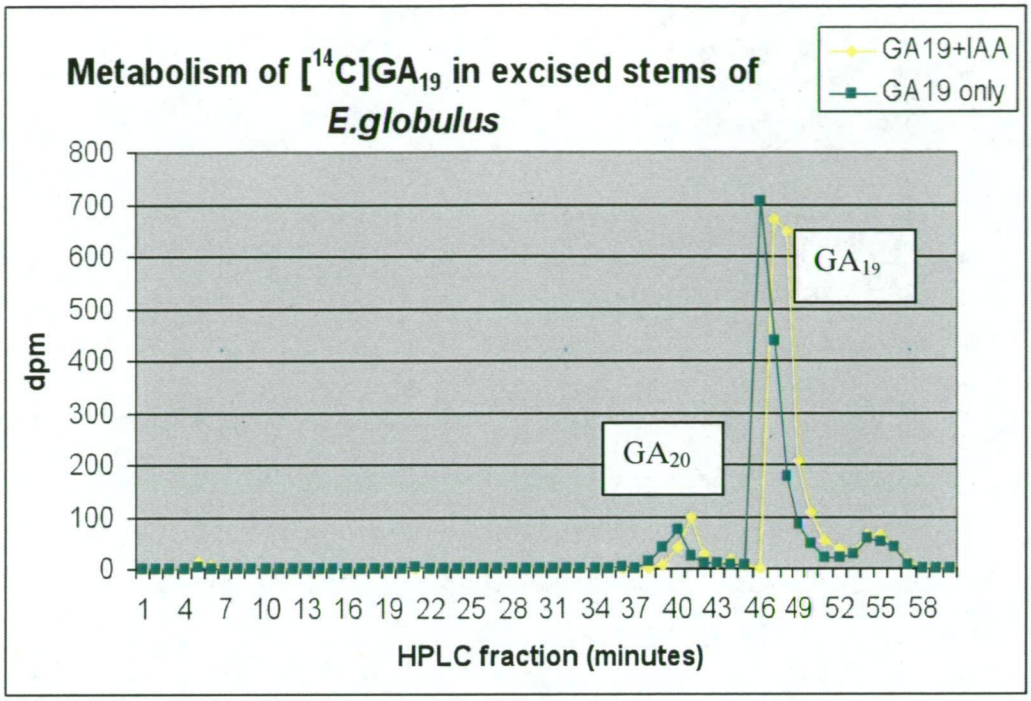


Figure 20.

[^{14}C]GA_{19} was fed to excised, elongating *E. globulus* seedling stem segments floating in MS media and its metabolites examined by radio-counting of methyl-ester HPLC fractions, n=1. Elongating *E. globulus* seedling stems were excised from the plant and floated in liquid MS media containing 400 000 dpm of [^{14}C]GA_{19} (specific activity 34 μCi/μmol) for 24 hours. IAA was added to one of the treatments at 5 μg/ml.

The lack of any significant difference in GA metabolism in the presence or absence of IAA is contrary to observations of excised *Pisum* stem segments (O'Neill and Ross 2002) and excised barley stems (Wolbang *et al.* 2004) in MS media containing similar hormone concentrations. In pea, IAA has been found to up-regulate the expression of the rate-limiting *PsGA3ox1* (Ross *et al.* 2000) and IAA action inhibitors have been shown to down-regulate both *PsGA3ox1* and *PsGA3ox2* in pea roots (Weston *et al.* unpublished data). Levels of bioactive GA_1 are also found to be increased or decreased accordingly in these systems.

Differing gene responses in other species

In aspen, where GA 20-oxiadation is rate limiting (Eriksson *et al.* 2000; Isrealsson *et al.* 2004), both *PttGA20ox1* and *PttGA20ox2* were found to be up-regulated by application of auxin in decapitated seedling stems. However, levels of bioactive GAs were unaffected after 24 hours, although GA_4 was moderately increased after 48 hours by this up-regulation of GA 20-oxidase genes (Bjorklund *et al.* 2007).

In the hybrid aspen seedling system, expression of either *PttGA2ox1* or *PttGA2ox2* was found to be down-regulated in response to application of either IAA or GA alone when compared to decapitated plants (Bjorklund *et al.* 2007). However, the overall effects of the treatments on GA 2-oxidase gene expression were not uniform. *PttGA2ox1* was found to have the highest expression level in intact plants and where both IAA and GA were applied together, while expression of *PttGA2ox2* expression was greatest in response to decapitation. The expression of *PttGA2ox2* was reduced by application of either or both IAA and GA (Bjorklund *et al.* 2007) and was low in intact plants. The effect of IAA application on GA biosynthesis and metabolism genes but not levels in trees suggests that their mechanisms for maintaining GA homeostasis may be more robust than herbaceous species.

O'Neill and Ross (2002) demonstrated a similar discrepancy in GA 2-oxidase gene response in decapitated pea. *PsGA2ox1* was found to be up-regulated in response to decapitation and down-regulated by the application of IAA, whereas *PsGA2ox2* was up regulated only when IAA was applied to decapitated stems. Frigerio *et al.* (2006) also showed differential effects of auxin treatment on the relative expressions of a suite of GA biosynthesis and deactivation genes in *Arabidopsis*. Two of four GA 20-oxidase genes and five of eight GA 2-oxidase genes were found to be up regulated by application of 50 μM NAA and one *GA 2-oxidase* was down regulated by the treatment. Of the genes with increased expression, *AtGA20ox1* was expressed 7-fold more than untreated, *AtGA2ox3* was expressed in excess of 280-fold compared with untreated levels and *AtGA2ox8* was expressed 39-fold higher in response to NAA.

These differing gene expression levels and responses indicates that there are probably separate homeostatic mechanisms in plants working together to maintain appropriate GA content. The varying levels of response, and in some cases opposing responses, of these GA biosynthesis and metabolism genes hints at independent regulation of the different genes by various environmental or experimental effects.

Differing metabolic rates

In these experiments, the proportion of metabolised/un-metabolised radio-labelled GA differed substantially between the different GAs that were applied. When GA_{19} was added to the media there was a 7:1 ratio of GA_{19} to GA_{20} in stems after 24 hours and when GA_{20} was added to the media there was a 1:1 ratio of GA_{20} to GA_{29}. When GA_1 was added there was an even greater proportion of metabolised substrate after 24 hours with a 1:3 ratio of GA_1 to GA_8 observed. All of these differences are consistent with strong homeostatic regulation of bioactive GA content of the stem as neither of the

applied GA precursors resulted in a measurable quantity of radio-labelled bioactive GA. When GA_1 was applied the majority of labelled GA present after 24 hours was the inactive metabolite GA_8.

It is possible that when [^{14}C]GA_{20} was added to the media a small proportion of labelled hormone was metabolised to GA_1, promoting the deactivation of the remaining substrate to GA_{29} by feedback mechanisms. Although there is no direct evidence that feedback mechanisms exist in *E. globulus*, their presence in both dicot and monocot herbaceous species suggests that these mechanisms are likely to operate in most, if not all higher plants.

In experiments where GA_1 was quantified from seedling stems, amounts present were below 1 ng/gFW^{-1}. Thus, addition of 1.9 μg of [^{14}C]GA_{20} (specific activity 55 μCi/μm) to the MS media could feasibly have boosted bioactive GA content 10-fold over normal levels in the stems while still constituting less than 1 % of the total available labelled GA pool. An increase in bioactive GA of this magnitude would presumably be sufficient to promote feedback regulatory steps if they are present and functional in these tissues. Feedback regulation is thought to be the most likely cause of the discrepancy between the effects of IAA on decapitated/defoliated seedling stems and the excised stems in MS media. Additionally, these experiments were all conducted over a 24 hour time period whereas many similar experiments are conducted over 2-8 hours. In previous work, longer time-frames resulted in increased production of deactivated GA compared to bioactive forms (O'Neill and Ross 2002). However, in these experiments applied GA_{20} was all deactivated to GA_{29} rather than via GA_1 to GA_8, indicating that at no point in the 24 hours were any significant quantities of bioactive GAs being produced.

Chapter 5- Effects of girdling and auxin application on IAA and gibberellin levels in tree trunks

Introduction

Both auxins and GAs are known to be present in the cambial region of trees including *E. globulus* (Hasan *et al.* 1994; Ridoutt *et al.* 1995) and it has been shown that these two hormones act synergistically in regulating the differentiation and subsequent development of cambial derivatives (Wareing *et al.* 1964; Wang *et al.* 1997; Kalev and Aloni 1998). The effects of IAA on GA levels, such as those demonstrated in herbaceous species and tree seedlings discussed in the preceding chapters, have not previously been examined in tree trunks or the vascular cambial region.

The growth-promotive synergies between IAA and GA in the vascular cambium were first reported by Wareing (1958) who applied either IAA, GA_3 or both to *Acer pseudoplatanus* seedlings and excised stems of *Poplus nigra v. italica* and *Fraxinus excelsior*. Application of GA alone was reported to promote cell division but not differentiation, while application of IAA alone promoted the formation of a small amount of differentiated xylem. When both hormones were applied together a wide zone of differentiated and apparently 'normal' xylem was formed (Wareing 1958). These effects of auxin and GA on cambial growth and differentiation have also been reported in the conifers *Larix decidua* (Wareing *et al.* 1964) and *P. sylvestris* (Wang *et al.* 1997) as well as the herbaceous species *Coleus blumei* and *Phaseolus multiflorus* (Wareing *et al.* 1964).

In hybrid aspen and *P. sylvestris* IAA has been shown to a have distinct radial concentration gradient across the vascular cambial region (Uggla *et al.* 1996; 1998; Tuominen *et al.* 1997). Additionally, in aspen GAs and GA biosynthesis and metabolism genes have also been shown to have radial gradients in levels/expression respectively (Isrealsson *et al.* 2005). The radial gradient of IAA is thought to provide positional information to the developing xylem in a similar manner to morphogenic fields in animals.

The metabolism of radio-labelled GAs in the vascular cambium has been studied in both *P. sylvestris* (Wang *et al.* 1996) and *E. globulus* (Ridoutt and Pharis 1998). Applied GAs were metabolised in both systems, and were also found to be mobile in *P. sylvestris*. Sampling was only undertaken from the area surrounding the application site in *E. globulus* thus there is as yet no information concerning the possibility of transport of GAs in this region of *E. globulus*.

Basipetal auxin transport has been well documented in herbaceous species and has also been documented in seedlings and young stems of trees, but not extensively studied in the trunks of larger trees. IAA has long been associated with the repression of lateral and epicormic buds, although it is likely not the only factor that influences bud break (Morris *et al.* 2005). In *Quercus robur* L., a greater number of epicormic buds broke below the site of partial girdles than was observed either above the girdles or at the same height in un-girdled trees. The promotion of epicormic bud break was also found to be dependent on season. Girdles cut in spring (April-May) were found to have a greater effect than girdles cut in summer (June or August). The effects of girdling were partially reversed by application of NAA Wignall *et al.* 1987). The effects of girdling on epicormic bud growth were attributed to a reduction of IAA levels below the sites of the girdles, suggesting that IAA is basipetally transported in the cambial region in this species. More recently, IAA levels have been found to be higher

immediately above the site of a wound in *Populus tremuloides* undergoing secondary growth and lower below the site. The area of reduced IAA levels increased longitudinally over time at about 4 mm/hour, consistent with the rate of polar auxin transport (Kramer et al 2008).

Applications of radio-labelled IAA to woody tissues have also suggested that it is basipetally transported in a similar fashion to transport in herbaceous stems. Tritiated IAA was found to be basipetally transported in excised stems of *Fagus sylvatica* L. and this transport was localized in the cambial region (Lachlund and Bonnemain 1984). Polar transport of [$^{13}C_6$]IAA was also suggested by Sundberg and Uggla (1998) in decapitated *P. sylvestris* seedlings. Labelled IAA contributed 90 % of the total stem IAA below the application site and this proportion of labelled/endogenous IAA was reduced at successive sampling points down the length of the stem. These results also suggest that there may also be some *de-novo* biosynthesis of IAA in this region.

It was suggested by Field (1974) that the centripetal movement of IAA applied to the bark of *Salix fragilis* was promoted by GAs, possibly by increasing cell membrane permeability. GAs have also been linked to increased polar IAA transport in hybrid aspen (Bjorklund *et al.* 2007) by up-regulating the IAA transport gene *PttPIN1*. However, the effect of GAs alone on the expression of *PttPIN1* was less than the promotion of this gene's expression by IAA although the combined effect of both hormones was greater than IAA alone.

Schrader *et al.* (2003) demonstrated radial expression profiles for six putative IAA transport genes and up-regulation by IAA of five of these genes in IAA deficient tissue. Bjorklund *et al.* (2007) reported that 83 % of genes induced by GAs were also induced by IAA in hybrid aspen seedlings, so it is quite likely that IAA transport genes are also inducible to some extent by GAs.

GA promotion of IAA transport genes might have a role in promoting polar IAA transport and/or maintaining the radial IAA gradient.

Recent work by Bjorklund *et al.* (2007) has also shown an effect of decapitation on IAA and GA levels in the woody stems of hybrid aspen seedlings. Decapitation was found to reduce both IAA levels and GA biosynthesis gene expression while application of IAA to decapitated seedlings promoted the expression of GA biosynthesis genes. However, levels of bioactive GAs were not reduced by decapitation. Application of IAA to decapitated plants resulted in an increase in levels of GA_4 after 2 days while GA_1 levels were unaffected by IAA application.

To further investigate both IAA transport in trees and the possibility that IAA levels influence levels of bioactive GAs, 8 year old *E. globulus* trees approximately 15 m tall were girdled at breast height. 2,4-D dissolved in ethanol was applied to girdled trees in lanolin paste to the bottom edge of the girdle. Lanolin/ethanol mixture was applied as a control treatment. IAA, 2,4-D and GAs were quantified from cambial scrapings both above and below the girdle.

Methods

Treatment and location

Trees ranging in age from six to eight years old (Figure 21) and harvested between 2004 and 2006 (depending when individual experiments were conducted over the course of the project) growing in a forestry coup (Forestry Tasmania TA003A, 43° 02'13" S, 147°54'24" E, approximately 300 m elevation) were treated by girdling with a chisel at breast height (1.3 m). The girdle was cut to a minimum depth of the mature xylem. Either 2,4-D (Sigma-Aldrich Co. D7299/PAL Chemicals Ltd. Dorking UK) in 500 µL

EtOH and 3-5 g Lanolin or EtOH and lanolin only were applied to the bottom surface of the girdle. 0.15 g of 2,4-D was found to be optimal for approximating un-girdled endogenous auxin levels in the zone below the girdle although uptake varied substantially between trees. The girdled site was then wrapped in aluminium foil and sealed at the top with adhesive tape, to minimise water entry and other disturbances. Pilot experiments revealed up to 2-fold variation in IAA levels between trees, but no difference in levels at heights of 1 m and 1.6 m. There was no substantial drop in IAA levels below the site of girdling after 48 hours, but when treatment was applied 7 days before harvest there was approximately 20-30 fold reduction in IAA levels below the girdle. This time interval was used for all subsequent experiments. Due to the variability between trees, each treated or control tree was paired with one nearby of similar height, girth, and crown size.

Figure 21.

Eucalyptus globulus trees typical of those used in these experiments, approximately 7 years old.

Harvest

Trees were felled with a chainsaw and stem sections to be harvested were transported back to the laboratory (2 hour journey). One section above and up to 3 sections below the site of girdling were stripped of bark (Figure 22) and the exposed cambial tissue was immediately and gently scraped off with a razor blade, weighed, and placed in cold (-20°C) 80 % (v/v) methanol (MeOH) containing 250 mg/L butylated hydroxytoluene (BHT). Harvested stem sections were 250 mm in length and began approximately 100 mm above or below the site of girdling to prevent contamination of tissue with exogenous 2,4-D. There was no gap between individual lower stem segments other than the separation from the girdle. Each tree harvested was 1 replicate. Harvest fresh weights varied between experiments due to changes in cambial moisture content and possibly also annual growth cycles. Extraction and analysis of hormones was as describes in chapter 2.

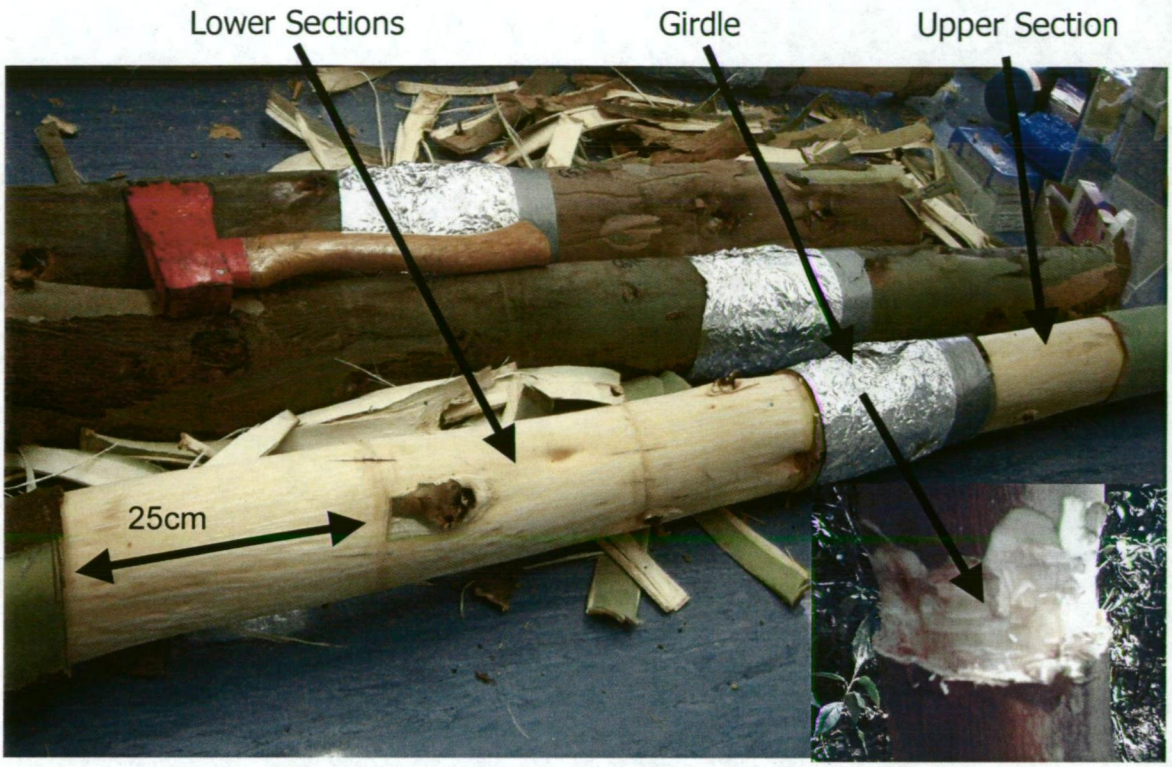


Figure 22.

Pilot girdling treatment applied with an axe, a chisel was used for later experiments (inset) and cambial harvest of *E. globulus* tree trunks (main). The exposed cambial region was scraped with a razor, weighed, and placed in cold 80 % MeOH with 250 mg BHT/L (see text for further details).

Results

Girdling had no significant impact (t-test, $P>0.05$) upon levels of IAA in the cambial region 2 days after treatment (Figure 23) but substantially and significantly (t-test, $P=0.019$) reduced IAA below the girdle after 7 days (Figure 24). The reduction in IAA levels was greatest in the stem section (25 cm sections) immediately below the girdle. Trees girdled in October (spring) were only deficient in IAA in the stem section immediately below the girdle, while trees girdled at any other time of year had reduced IAA for all of the sections below the girdle. The reduction in levels of IAA was consistent across all girdled trees regardless of 2,4-D application. Levels of IAA above the girdle were similar to intact trees.

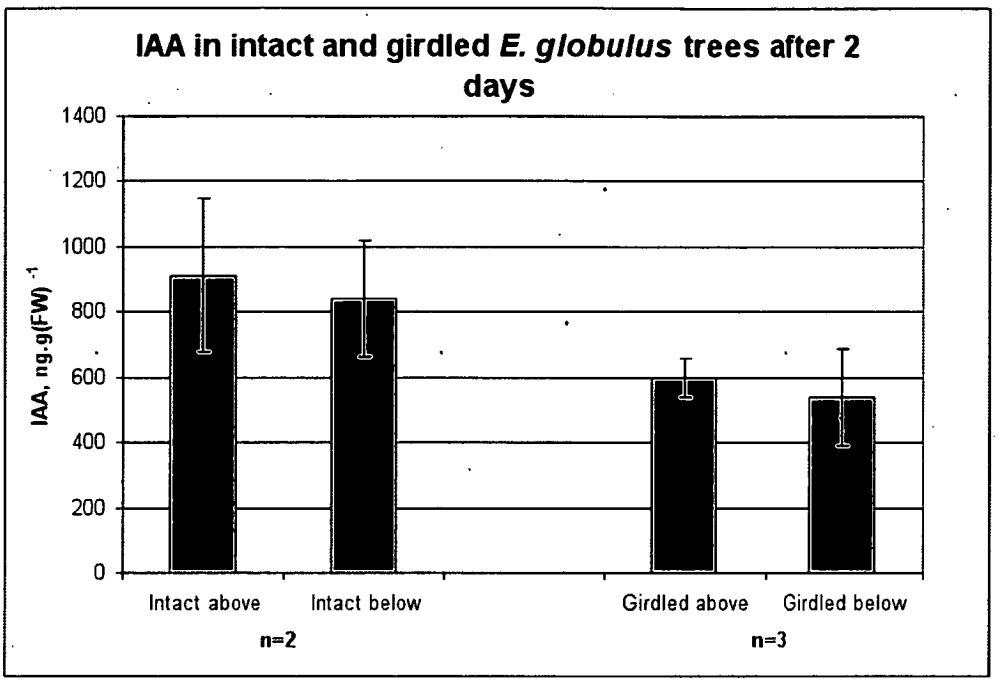


Figure 23.

Effect of girdling on the levels of IAA in the cambial zone of *E. globulus* above and below a girdle cut 2 days previously. Hormones were extracted from the entire circumference of the tree over 250 mm longitudinal sections. There was a 100 mm buffer on the upper and lower sides of the girdle to prevent contamination. 'Girdled above' was the section above the girdle, 'Girdled below' was the section below the girdle, above and below in intact trees is the position at the same height as that in girdled trees. Bars show standard error.

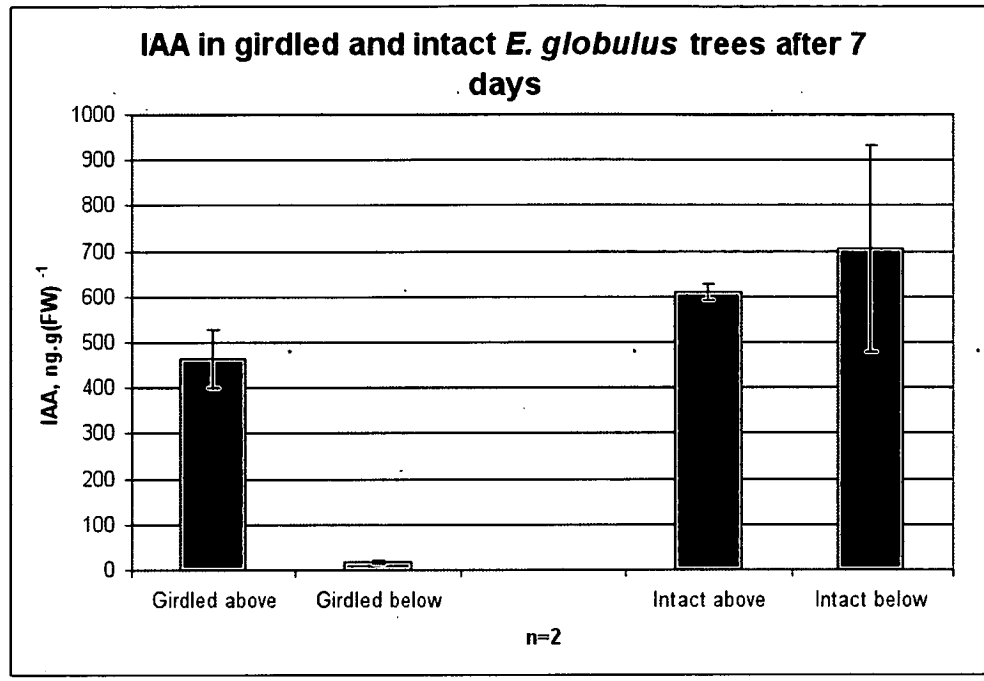


Figure 24.

Effect of girdling on the levels of IAA in the cambial zone of *E. globulus* above and below a girdle cut 7 days previously. Hormones were extracted from the entire circumference of the tree over 250 mm longitudinal sections. There was a 100 mm buffer on the upper and lower sides of the girdle to prevent contamination. 'Girdled above' was the section above the girdle, 'Girdled below' was the section below the girdle, above and below in intact trees is the position at the same height as that in girdled trees. Bars show standard error.

In contrast to the situation for IAA, there was no substantial effect of either 2,4-D or girdling on levels of GA_{20} in *E. globulus* trunks. Although trees were girdled in January (summer) GA_{20} levels were reduced approximately 2-fold below the girdle (compared with above the girdle, Figure 25) this result is not statistically significant (n=1 in this experiment). When the experiment was repeated in March (autumn) there was no reduction in GA_{20} levels below the girdle compared to levels above the girdle for either girdled-only trees or 2,4-D treated trees (Figures 27 and 28). A third repetition of the experiment in October (Spring) actually showed a reduction in GA_{20} above the girdle, but this was only significant ($P \leq 0.05$) in 2,4-D treated trees (Figures 29 and 30). 2,4-D uptake was not consistent with season. In March and October harvests, 2,4-D levels were not as high in the stem segments below the girdle as they were in the January harvest (Figures 26, 28 and 30) despite the amount and application method being the same.

It was not possible to obtain complete sets of data for the other GAs of interest in these experiments. Ongoing problems with impurities and in many cases very low endogenous levels prevented accurate quantification. GA_8 was below detectable levels from cambial tissue and GA_{29} was beset by the same problems as GA_1 with both low endogenous levels and interference on GC-MS by impurities. GA_{19} was quantified from some cambial extracts and was generally unaffected by either girdling or 2,4-D application.

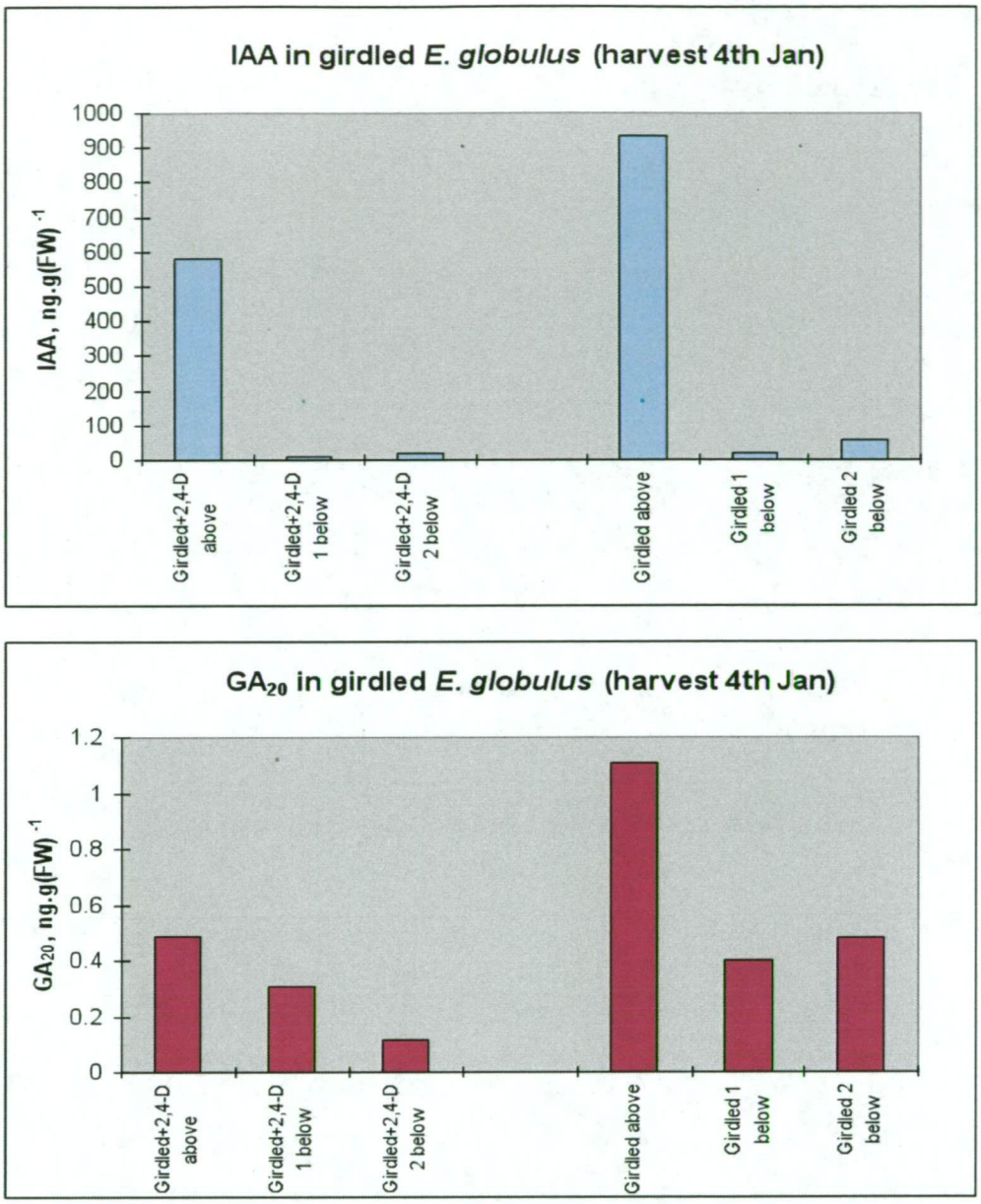


Figure 25.

Effect of girdling on the levels of IAA and GA_{20} in the cambial zone of *E. globulus*. Hormones were extracted from the entire circumference of the tree over 250 mm longitudinal sections. There was a 100 mm buffer on the upper and lower sides of the girdle to prevent contamination. 'Girdled above' was the section above the girdle, 'Girdled χ below' were adjoining sections numbered sequentially beginning below the girdle. Either 2,4-D in ethanol and lanolin or ethanol and lanolin only were applied when the girdle was cut 7 days before harvesting, n=1.

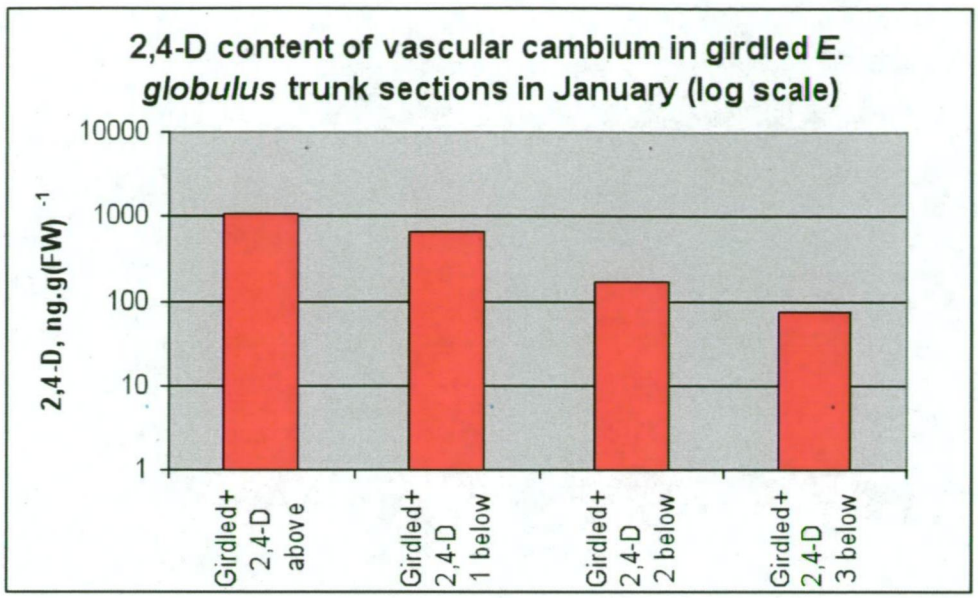


Figure 26.

2,4-D content of sections of vascular cambium above and below a girdle cut 7 days previously. 2,4-D was applied around the girdle in ethanol and lanolin when the girdle was cut. 2,4-D levels were measured in a cambial scrape of a whole circumference, 250 mm longitudinal section of *E. globulus* trunk above the girdle and in 3 sections below the girdle, n=1. No 2,4-D was detected in truck sections to which it was not applied (data not shown).

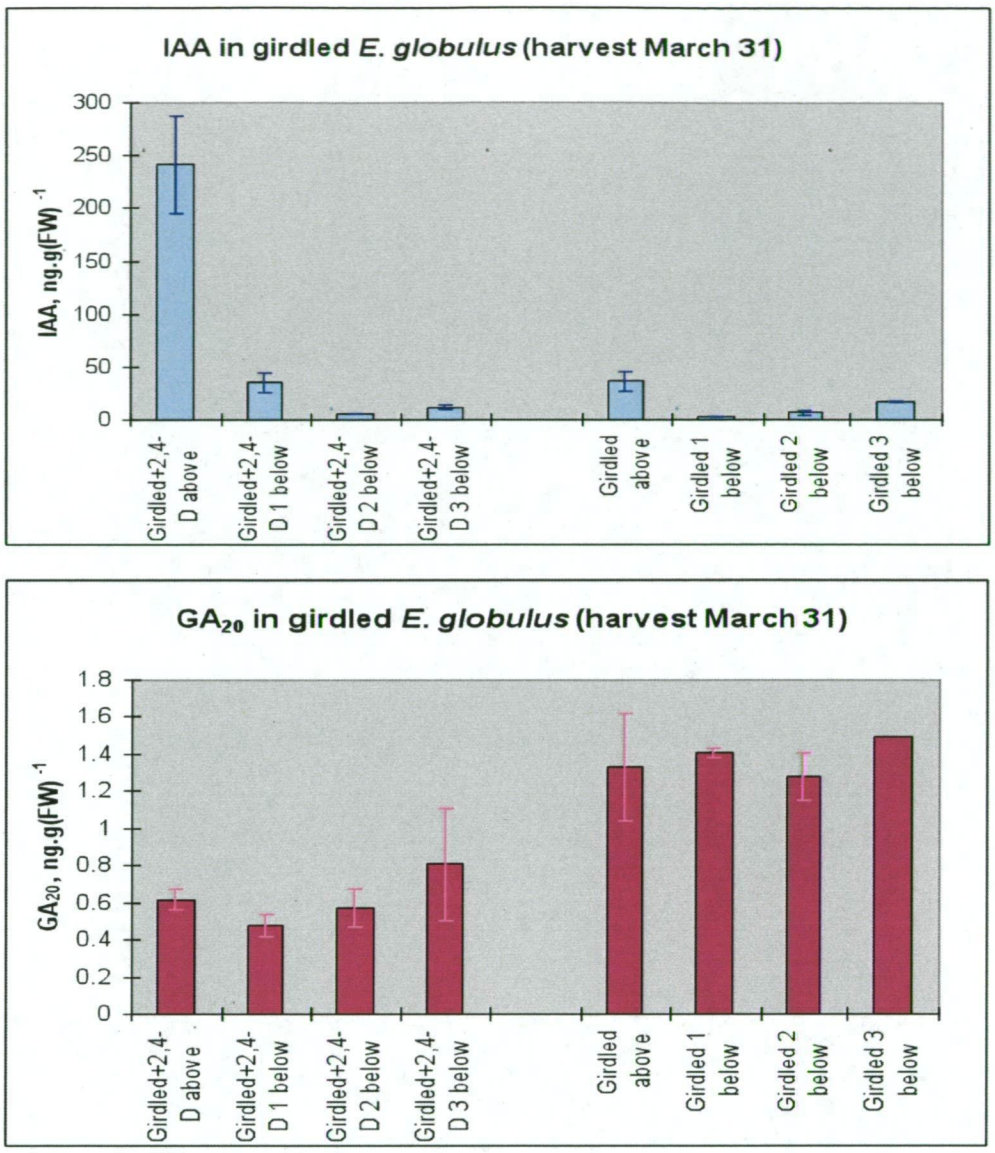


Figure 27.

Effect of girdling on the levels of IAA and GA_{20} in the cambial zone of *E. globulus*. Hormones were extracted from the entire circumference of the tree over 250 mm longitudinal sections. There was a 100 mm buffer on the upper and lower sides of the girdle to prevent contamination. 'Girdled above' was the section above the girdle, 'Girdled χ below' were adjoining sections numbered sequentially beginning below the girdle. Either 2,4-D in ethanol and lanolin or ethanol and lanolin only were applied when the girdle was cut 7 days before harvesting. Bars indicate standard error and n=2 except for GA_{20} girdled 3 below where n=1; each tree is a replicate.

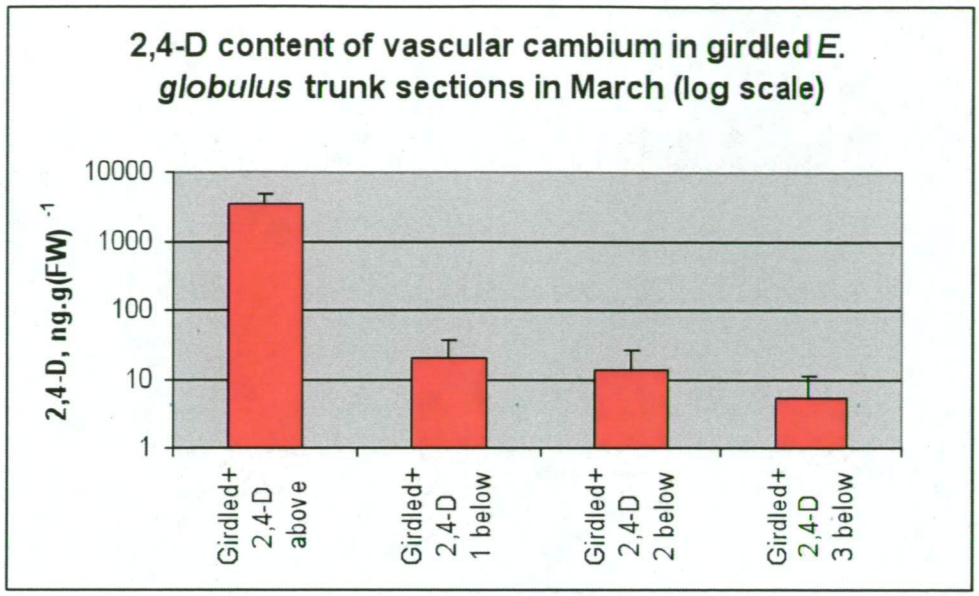


Figure 28.

2,4-D content of sections of vascular cambium above and below a girdle cut 7 days previously. 2,4-D was applied around the girdle in ethanol and lanolin when the girdle was cut. 2,4-D levels were measured in a cambial scrape of a whole circumference, 250 mm longitudinal section of *E. globulus* trunk above the girdle and in 3 sections below the girdle. Bars indicate standard error and n=2, each tree is a replicate. No 2,4-D was detected in truck sections to which it was not applied (data not shown).

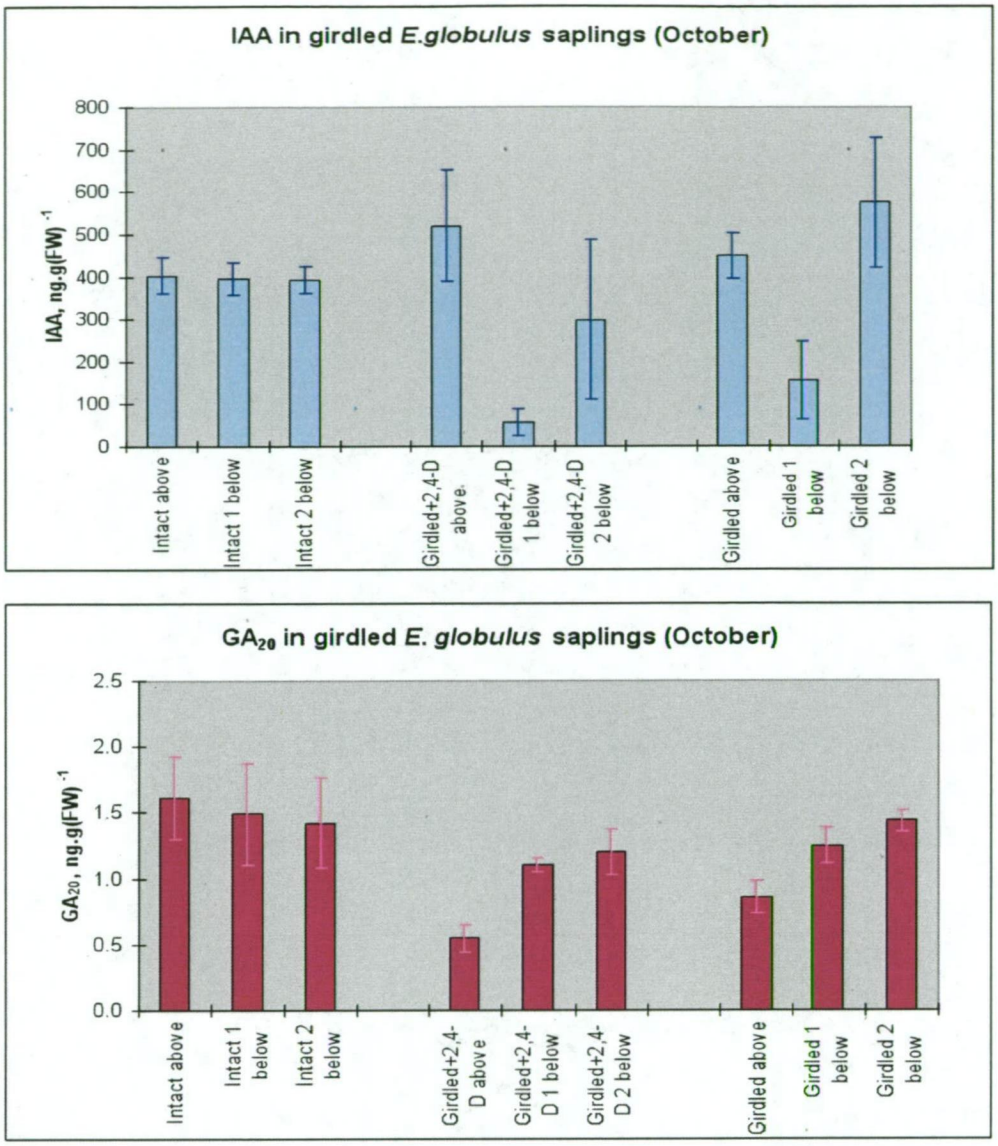


Figure 29.

Effect of girdling on the levels of IAA and GA_{20} in the cambial zone of *E. globulus*. Hormones were extracted from the entire circumference of the tree over 250 mm longitudinal sections. There was a 100 mm buffer on the upper and lower sides of the girdle to prevent contamination. 'Girdled above' was the section above the girdle, 'Girdled χ below' were adjoining sections numbered sequentially beginning below the girdle. Either 2,4-D in ethanol and lanolin or ethanol and lanolin only were applied when the girdle was cut 7 days before harvesting, bars indicate standard error and n=3, each tree is a replicate.

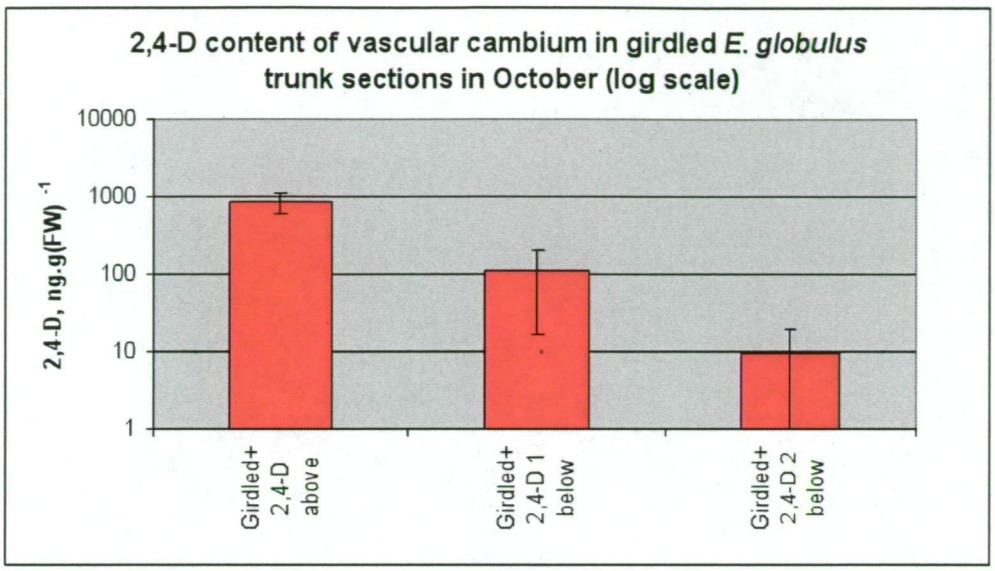


Figure 30.

2,4-D content of sections of vascular cambium above and below a girdle cut 7 days previously. 2,4-D was applied around the girdle in ethanol and lanolin when the girdle was cut. 2,4-D levels were measured in a cambial scrape of a whole circumference, 250 mm longitudinal section of *E. globulus* trunk above the girdle and in 2 sections below the girdle. Bars indicate standard error and n=3, each tree is a replicate. No 2,4-D was detected in truck sections to which it was not applied (data not shown).

Discussion

Auxin

Girdling substantially reduces IAA in the stem below the girdle after 7 days but has no discernable effect after 2 days. This is substantially different from herbaceous species and seedlings where large reductions in IAA can be observed in 2-48 hours depending on the experimental system used. Although the sections measured in these experiments were longer (250 mm) than those generally used in other systems (50-100 mm typically) the auxin reduction caused by girdling took longer to eventuate than expected. This suggests that IAA transport in the cambial region of tree trunks might be slower than in younger or herbaceous stems. This is the first direct evidence for basipetal auxin transport through the vascular cambial region in *Eucalyptus*.

When the experiment was performed in October, there was only a reduction in IAA levels in the stem section immediately below the girdle. This indicates that, if the reduction in IAA levels is due to an interruption of basipetal transport, the rate of transport is less in spring. Additionally or alternatively, the stem might be producing some IAA *in situ* and a higher rate of production in spring would also produce this result.

It was suggested by Sundberg and Uggla (1998) that in situ synthesis of IAA occurred in decapitated stems of *P. sylvestris* as radio-labelled IAA applied to the cut stump of decapitated seedlings was found to be diluted with a higher proportion of unlabeled IAA at sampling points further down the stem. However, Wignall *et al.* (1987) reported that partial girdles cut into *Quecus robur* L. promote epicormic bud sprouting, and that this was probably due to an interruption of IAA transport (although IAA was not directly measured). It has also been reported that girdling interrupts the movement of a wave of

radio-labelled IAA applied to shoots of *Abies balsamea* (Little 1981). The effects of girdling on levels of IAA are also comparable to the effects of either NPA or methyl-2-chloro-9-hydroxyfluorene-9-carboxylic acid (CF) (auxin transport inhibitors) when applied in a ring around the stem of 1 year old *P. sylvestris* seedlings (Sundberg *et al.* 1994). It remains unclear which (if not both) of these hypotheses regarding the source of IAA in the vascular cambium of tree trunks is correct.

Gibberellins

GA_{20} levels were reduced below the girdle compared to levels above the girdle during January and reduced above the site of girdling in October. There was no effect of girdling on GA_{20} levels in March. Seasonal effects on GA levels have been previously reported in *E. globulus* (Matyczek 1995) and day length has been shown to affect *GA 20-oxidase* activity in hybrid aspen (Eriksson and Moritz 2002). However, the differences in GA_{20} levels observed were small and most were not statistically significant. Levels of GA_{20} were consistent reasonably throughout the year. It is not likely that GA_{20} levels are affected by either girdling, 2,4-D or season in *E. globulus* trunks.

GA_1 was found to be below the limit for accurate quantification in the October experiment, signifying that its levels were well below those previously reported for the vascular cambium of *E. globulus* (0.27 ng/gFW^{-1} and 0.3 ng/gFW^{-1}, Hasan *et al.* 1994; Ridoutt *et al.* 1995 respectively). Successful quantification of downstream GA_{20} products and metabolites would have provided some interesting insight into this unexpected result.

The quantification of gibberellins was hampered by the relatively small amount of tissue available for analysis. To collect tissue from a larger area of the trunk would have compromised the level of detail of the experiment, and

to pool tissue from a greater number of trees would have required more resources than were available for the project. The methodology also had to be adapted to be suitable for processing a large number of samples.

General Discussion

The effects of girdling on auxin and GA levels were studied for the first time in the vascular cambial region of tree trunks. Girdling was found to reduce IAA levels in the vascular cambium below the girdle, similar to the reduction in IAA levels in decapitated/defoliated stems of seedling and herbaceous species. Girdling was also found to have variable or no effect on GA levels, although problems with impurities and/or very low levels made direct, accurate quantification of bioactive GAs impossible. There was no apparent effect of auxin application to girdled trees on any of the GAs measured in these experiments, contrary to the promotion of GA biosynthesis and levels reported in several herbaceous species.

Seedlings

In several herbaceous species IAA has been shown to, or suggested to, promote GA biosynthesis (Ross 1998; Ross *et al.* 2000; Wolbang and Ross 2001; O'Neill and Ross 2002; Wolbang *et al.* 2004; Frigerio *et al.* 2006; Yin *et al.* 2007). Whilst the biosynthetic steps affected in different species are not necessarily the same, levels of the relevant bioactive GAs are increased by IAA application to decapitated/defoliated stems, or stems deficient in auxin through other means.

The effects of IAA levels on the expression of GA biosynthesis genes have also been studied in herbaceous species. In pea, IAA has been shown to up-regulate the expression of, and increase the transcript abundance of, the rate limiting gene *PsGA3ox1* (O'Neill and Ross 2002). IAA has also been shown to down-regulate GA de-activation genes (Ross *et al.* 2000). However, in *Arabidopsis* and hybrid aspen not all GA biosynthesis and deactivation genes are equally affected by treatments or environment (Frigerio *et al.* 2006; Bjorklund *et al.* 2007). The differences between gene expression levels and

actual hormone concentrations in plant tissue highlight the hazards of extrapolating gene expression level for actual hormone levels. It must also be noted that even hormone levels are not the ultimate indicator of hormone activity as perception of, and sensitivity of cells to hormone levels also contributes to overall hormone activity. In the case of GAs, which promote growth by accelerating the degradation of the growth repressing DELLA proteins, the levels of these and the responsiveness of cells to their action would also have to be quantified to obtain a truly accurate "GA response" estimate. Hence, the lack of response of GA levels to auxin application in tree trunks does not preclude there being any GA response to auxin. A change in GA gene expression (possibly dampened by homeostatic mechanisms) might not necessarily lead to a detectable change in GA levels. It is also entirely possible that auxin affects GA perception or GA response rather than GA levels in the vascular cambium. Changes in these would not be detectable through observation of GA levels except where feedback mechanisms may impact upon these.

Previous work with IAA and GAs in trees suggests that these two hormones act synergistically to promote growth in the vascular cambial region (Wareing 1958; Wareing *et al.* 1964; Digby and Wareing 1966; Field 1974; Wang *et al.* 1995; Kalev and Aloni 1998). Decapitation has been shown to reduce IAA, but not GA levels in seedling stems, while defoliation reduces both IAA and GAs (Wang *et al.* 1997; Bjorklund *et al.* 2007). Application of IAA to decapitated seedling stems alters the expression of some GA biosynthesis and metabolism genes in hybrid aspen seedling (Bjorklund *et al.* 2007), resulting in a net increase in GA_4 after 2 days, but no effect on GA_1 levels. Whilst this increase in active GA is not as large as seen in herbaceous species, it must be noted that herbaceous species typically have reduced bioactive GA content when decapitated.

Although not all GAs were able to be accurately quantified from seedlings in this project, the GAs that were quantified respond in a similar manner to decapitation/defoliation and auxin application as other species when subject to similar treatments and IAA application. This suggests that the promotion of GA biosynthesis by IAA might be common across all elongating primary shoots in higher plants.

Metabolism-seedlings discrepancy

There was a discrepancy between the results obtained when feeding radio-labelled GAs to excised *E. globulus* seedling stems and those obtained from decapitated seedlings of *E. globulus*. The tissues used and the conditions under which it was grown were very similar between these experiments. Also, the reduction in IAA levels by the respective treatments and the recoveries in IAA levels by the relevant application were similar in magnitude between the two experiments. Despite these similarities, none of the radio-labelled GAs applied were metabolised differently in the presence or absence of IAA in excised stems while in seedlings, IAA levels did affect the levels of GA_{20} and GA_8 (and probably GA_1).

Feedback regulation of bioactive GA levels has been demonstrated in pea (Martin *et al.* 1996; Ross *et al.* 1999), and it is thought likely that a similar mechanism would operate in *E. globulus* although this has yet to be demonstrated. It is thought likely that the difference in GA metabolism was due to the different levels of GAs available in these systems. No GAs were added to the seedling experimental system, while quantities of GA several orders of magnitude higher than typical endogenous content were added to the liquid MS media. It is possible that some form of homeostatic mechanism promoted the conversion of these high levels of exogenous radio-labelled GAs to inactive forms. An alternative and/or additional possibility is

that the relative abundance of radio-labelled GA metabolites could have swamped any changes in bioactive GA levels due to IAA in these experiments.

Trees

IAA was reduced below the site of girdles cut in the trunk of *E. globulus* trees. This reduction is similar in magnitude to the reductions in IAA observed in decapitated/defoliated seedling stems of *E. globulus* as well as seedlings and stems of other tree species. It is also similar to the reductions observed in herbaceous species that have been decapitated and/or defoliated. This finding supports the hypothesis of Wignall *et al.* (1987) that girdling interrupts the basipetal flow of IAA in tree trunks (based on observations of bud break above and below partial girdles) with empirical measurements of IAA levels above and below girdles. It is also in agreement with the findings of Kramer *at al.* (2008) that IAA levels are reduced below the site of wounding in *Populus tremuloides*.

The reduction in GA_{20} levels observed below the site of the girdle in trees in the January experiment is comparable to the effects on GA_{20} levels of the decapitation/defoliation treatment in seedlings, as well as decapitated herbaceous species. However, the January harvest consisted of a single replicate so this result is not statistically significant. In the March and October replicates of the experiment there was no decrease in GA_{20} below the site of the girdle. These results suggest that IAA might not be an important regulator of GA_{20} levels, and possibly not bioactive GAs, in the vascular cambial area.

The lack of effect of 2,4-D application to girdled tree trunks is not directly comparable to any other previous results from other species/experimental systems. The closest analogue to this study is the work of Bjorklund *et al.*

(2007) who applied IAA to decapitated hybrid aspen. In this work, applied IAA did increase the levels of GA_4 after 2 days while GA_1 was not affected. It should be noted that bioactive GA levels were not reduced by the decapitation treatment in this experimental system.

The lack of effect of 2,4-D on GA levels is unlikely to be due to a lack of auxin activity of this compound, as it has been shown to be capable of promoting GA biosynthesis in pea (O'Neill and Ross 2002). Applied 2,4-D was often not found to be present in the stem sections below the girdle at quantities thought to be sufficient to promote GA biosynthesis (a level approximate to IAA levels in intact plants), although even when it was present at an adequate level there was no discernable effect. The time-frame of these experiments was longer than that typically used in other IAA/GA interaction experimental systems. This was necessary to generate a sufficient reduction in IAA below the girdle. However, IAA/GA interaction experiments are more typically conducted over a 24-48 hour time-frame and it is possible that beyond this, the effects of IAA reduction or application on GA levels are overridden by other mechanisms.

Spatial separation

In the cambial region of aspen trees, GA biosynthesis genes have been found to be expressed predominantly in the phloem and in the expanding xylem (Isrealsson *et al.* 2005) whilst IAA levels were found to highest in the vascular cambium itself (Tuominen *et al.* 1997; Uggla *et al.* 1998). If auxin is an important promoter of GA biosynthesis in this region, it would be expected that GA biosynthesis genes would be most active where the IAA content is highest. Quantification of GAs on a radial gradient through the vascular cambial region also showed spatial separation of the peaks of IAA and GA concentrations. The immediate bioactive GA precursors GA_{20} and GA_9 were found to be concentrated in the phloem, while the bioactive

gibberellins GA_1 and GA_4 were concentrated in the zone of expanding xylem (Isrealsson *et al.* 2005). It was suggested that GA precursors were likely to be transported through the cambial region in the rays, and that bioactive GAs were synthesised in the developing xylem.

Both the spatial separation of, and the lack of any promotive (or indeed any) definite and consistent effect of auxin on GA_{20} levels in the cambial region suggests that auxin does not substantially promote GA biosynthesis in the vascular cambial region of tree trunks. It cannot be determined from the results of this study that auxin has no effect at all on GA levels or biosynthesis in the vascular cambium, but it is thought likely that it is not one of the more important regulators of GA biosynthesis or content in this region. It is also possible that the relative distributions of IAA and GA are not as significant as it has previously been thought. Many of the genes regulated by GA in hybrid aspen seedlings were shown to also be inducible by IAA (Bjorklund *et al.* 2007). In light of this finding, the relative concentrations of IAA and GA across the cambial region may only be significant to the extent of the few genes that are not induced by either hormone and the developmental roles these genes might have in their respective tissues.

General

This disparity between the effects of IAA on GA biosynthesis and levels in herbaceous species and seedlings compared to those observed in tree trunks may be due to differences between the growth of primary and secondary tissues. GAs have been strongly associated with the promotion of cell elongation in developing tissue. Although cellular elongation would be expected to be occurring in both young shoots (primary growth) and vascular cambial derivatives, both the proportion of cells elongating in the cambial region and possibly the degree of elongation occurring might be less.

When examining the differences in the results of the seedling and tree experiments, it is important to note that the tissues used for hormone analysis in these two experiments are not necessarily directly comparable. A more direct comparison would be between the procambium of the vascular bundles in primary shoots and the vascular cambium of trees. However, apart from the difficulty of isolating a sufficient quantity of procambium from seedling stems to extract hormone from, the analysis of whole stems has the benefit of allowing a more direct comparison between seedling stems and those of herbaceous species.

Experimental Problems

There were many technical problems encountered during the course of these experiments, reducing the amount of data produced and preventing some of the main aims of the project from being addressed with the desired weight of evidence.

Problematic compounds in the samples that interfered with GC-MS analysis were relatively consistent within individual experiments but not between experiments. This suggests that levels of these compounds are affected strongly by environmental factors. Although season is probably the largest factor influencing field grown trees, and may also have had some impact on seedlings, it also cannot be ruled out that genetics played a role in the levels of these compounds. The BPX-608 GC column used in the later experiments of the project did separate these compounds from the GAs of interest, but GA levels were found to be below the limits for accurate quantification in these experiments.

An alternative approach to direct quantification of GAs would be to monitor gene expression, as has been done in some other herbaceous species. The expression of GA biosynthesis genes has been shown to be indicative of

levels of product GAs in pea, and this method might be a better alternative to direct quantification of GAs in eucalyptus. However, as far as GA levels are concerned GA gene expression is only an indication, as different GA biosynthesis genes can be differently regulated by the same stimulus (Bjorklund *et al.* 2007). It would also be of interest to apply exogenous GAs to eucalyptus seedlings. This was not done as part of these experiments due to the danger of contaminating common facilities with large amounts of GA. Gibberellin biosynthesis inhibitors are already being used in commercial eucalyptus breeding programs while gibberellins themselves have not been utilised commercially.

A further complicating factor related to the influence of auxins on levels of GA in tree trunks was that the application of 2,4-D to girdled trees in a lanolin ring did not reliably introduce a consistent quantity of 2,4-D into the cambial region. Also, the concentration of 2,4-D was always in excess of an order of magnitude higher above the site of girdling than below it. Only in a few trees was the level of 2,4-D in the same range as that of endogenous IAA in intact trees in the segment below the girdling. This distribution pattern was unexpected, and it is interesting that 2,4-D was predominantly found in the region above the girdle. It has been suggest previously that 2,4-D is a substrate for the IAA influx carrier but not IAA efflux carriers (Morris and Robinson 1998). Its dispersion in plants is not necessarily analogous to that of IAA but the ability of 2,4-D to promote GA biosynthesis was shown by O'Neill and Ross (2002).

IAA was not used in these experiments as the remoteness of the site was not conducive to frequent re-application (which would presumably be required as IAA can be more easily conjugated/metabolised than 2,4-D). It was also desirable to use an auxin other than IAA for these experiments to prevent contamination of the laboratory/equipment with high levels of this hormone.

Overall, decapitation/defoliation treatment reduced IAA, GA_{20} and GA_8 in seedling stems, while application of IAA to decapitated/defoliated stems increased the levels of IAA, GA_{20} and GA_8 in these stems. Girdling of tree trunks reduced IAA levels in the cambium below the girdle after 7 days but had no significant impact on IAA levels after 2 days. There was no substantial effect of girdling or 2,4-D application on levels of GA_{20} in the vascular cambium.

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